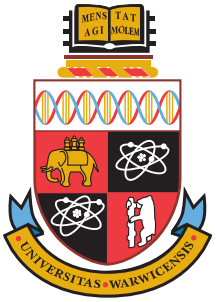

The downy mildew effector HaRxL21 suppresses immune responses of *Arabidopsis thaliana*

by

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Declaration

I confirm that my thesis has been prepared in accordance with the University guidelines on the presentation of a research thesis for the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted in any previous application for any degree. The work in this thesis has been undertaken by myself except where otherwise stated.

Abstract

The oomycete *Hyaloperonospora arabidopsidis* (*Hpa*) is the causal agent of downy mildew of *Arabidopsis thaliana*; a system that can be used as a model for the study of plant-pathogen interactions. In order for successful colonisation, biotrophic pathogens such as *Hpa* suppress or evade plant defences through secretion of effector proteins into the plant to manipulate and disrupt the host immune system. Alignment of oomycete effector proteins has revealed a conserved amino acid sequence at the N-terminus with the consensus sequence RxLR (arginine, any, leucine, arginine), thus allowing the use of Bioinformatic approaches to identify putative effector proteins in the *Hpa* genome.

Studying effector action and their targets in the host may help elucidate important components of the plant defence response, eventually leading to more durable crops. Expression of the *Hpa* effector HaRxL21 *in planta* has been shown to alter host susceptibility to *Hpa*, *Botrytis cinerea* and *Pseudomonas syringae*. Here the interaction targets of HaRxL21 are presented and interaction with the transcriptional co-repressor TOPLESS (TPL) has been validated *in planta* using BIFC and Co-IP. Using deletion and mutation analysis, the specificity of the interacting protein domains has been identified as between the CTLH domain of TPL and Leucine residues within the EAR motif of HaRxL21. Microarrays have revealed effects of HaRxL21 on host transcription, particularly up regulation of genes involved in ABA signalling and a decreased induction of SA-responsive genes upon SA induction. Finally, work has been carried out to determine the biochemical function of HaRxL21, showing an increased stability of TPL in the presence of this effector.

Abbreviations

ABA Abscisic Acid

Ade Adenine

B. cinerea *Botrytis cinerea*

BIFC Bimolecular fluorescence complementation

BIFP BIFC *in Planta*

cDNA Complementary DNA

CFU Colony forming units

CTLH C-terminal to LisH

DAMP Damage-associated molecular pattern

DAPI 4',6-diamidino-2-phenylindole

DNA Deoxyribonucleic acid

EAR Ethylene-responsive element binding factor-associated Amphiphilic Repression (motif)

E. coli *Escherichia coli*

ET Ethylene

ETI Effector Triggered Immunity

GFP Green fluorescent protein

His Histidine

Hpa *Hyaloperonospora arabidopsidis*

HR Hypersensitive response

JA Jasmonic Acid

Leu Leucine

LisH Lissencephaly type-1-like homology (motif)

MAMP Microbe-Associated Molecular Pattern

Nb *Nicotiana benthamiana*

OBE OBERON

pAD pDEST-AD

PAMP Pathogen-Associated Molecular Pattern

PCR Polymerase Chain Reaction

pDB pDEST-DB

PFO *Pseudomonas fluorescens*

Pi *Phytophthora infestans*

PIG *PAMP-induced gene*

Pst *Pseudomonas syringae* pv. *tomato*

P. syringae *Pseudomonas syringae*

PTI PAMP-triggered immunity

RFP Red fluorescent protein

RNA Ribonucleic acid

RT Reverse transcription

RT-PCT Reverse transcription polymerase chain reaction.

RXLR arginine, any, leucine, argenine (motif)

SA Salicylic Acid

SAR Systemic Acquired Resistance

SC Synthetic complete (media)

SDM Site Directed Mutagenesis

Ser Serine

SNP Single Nucleotide Polymorphism

SUMO Small Ubiquitin-like Modifier

SWAP SUPPRESSOR OF WHITE APRICOT

TCP TEOSINTE BRANCHED1, CYCLOIDEA, and PCF

TPL TOPLESS

TPR TOPLESS-RELATED

Trp Tryptophan

TTSE Type III secreted effector

Y2H Yeast-2-Hybrid

YFP Yellow fluorescent protein

Chapter 1

Introduction

1.1 Oomycetes as plant pathogens

Oomycete pathogens are responsible for many devastating diseases of agricultural crops as well as ornamental and native plants. Within the oomycetes are many important genera of plant pathogens such as *Albugo*, *Bremia*, *Peronospora* and *Plasmopora* that cause downy mildews and white rusts on several crops such as grapevine (Kamoun, 2003), as well as over 100 *Pythium* species which cause root rot on many glasshouse crop plants (Kamoun, 2003; Coates and Beynon, 2010). Also existing within the oomycetes are over 60 species in the genus *Phytophthora* which can cause large scale damage to many important crop species including potatoes, tomatoes, soybeans, peppers and alfalfa (Kamoun, 2003). *Phytophthora infestans (Pi)* is the causative agent of late blight of potato and tomato, resulting in the deaths of 1.25 million people during the Irish potato famine of 1845 (Agrios, 2005) and remaining a contemporary problem; global annual losses due to late blight of potato have been conservatively estimated to be €4.8 billion (Haverkort et al., 2008). Another *Phytophthora* pathogen, *Phytophthora ramorum* is resposible for causing sudden oak death in the USA in the late 20th century and currently causing epidemics on UK larch populations (Grünwald et al., 2012). Continued research into oomycete pathogens is therefore necessary for food security as well as the protection of ecosystems and economic interests. Despite this, Sheehy et al. (2008) show that the money spent annually on diseases such as malaria or HIV/AIDS far exceeds that spent on malnutrition, although many more people are affected by malnutrition (Table 1.1).

The emergence of pathogens such as *Phytophthora andina* (a hybrid of an unknown *Phytophthora* species with *Pi* that is able to infect new hosts) (Goss et al., 2011) exemplifies the need for an understanding of how these pathogens manipulate and infect their host

Table 1.1: Challenges to human health and the research budgets spent on addressing them.

Problem	Humans affected (millions)	Number of deaths each day	Money spent annually on research (million US$)	Spending per death (US$)
Malaria	400	5500	323	164.96
HIV/AIDS	40	8200	550	188.41
Malnutrition	854	2500	0.75	0.84

Challenges poised to humans in the 21st century and annual money spent on research aimed at solving them. Adapted from (Sheehy et al., 2008).

plants if we are to manage challenges poised in the future.

The oomycetes are a group of eukaryotes which have been described as 'fungus-like' although they belong to the kingdom Stramenopiles and are more closely related to brown algae and diatoms than higher fungi (Hardham et al., 1994; Birch et al., 2006). Oomycetes and fungi share several characteristics such as filamentous vegetative growth, production of feeding structures called haustoria, mycelia and the formation of spores (Latijnhouwers et al., 2003) however there are several important differences. Oomycete hyphae are coenocytic and therefore unlike the septate hyphae of fungi are not divided into cell-like units containing a nucleus. Another major difference is that unlike fungi, the cell walls of oomycetes have little or no chitin and are cellulose based (Hardham et al., 1994). This means that chitin synthase inhibitors (such as Nikkomycin and Polyoxin D (Gaughran et al., 1994; Endo et al., 1970)) have no inhibitory effects (Latijnhouwers et al., 2003) and can therefore not be used as a control agent against oomycetes. Another major target of fungicides is sterol synthesis; triazole fungicides target the biosynthesis of the fungal-specific sterol ergosterol. Because many oomycetes are sterol prototrophs and therefore do not synthesize ergosterol, these fungicides cannot be used to control diseases caused by these pathogens (Gaulin et al., 2010), therefore understanding the pathogenicity of these organisms in order to develop new methods of control is critical.

1.2 Plant defence against disease

Plants have many layers of defence which aid them in avoiding the frequent colonisation attempts by phytopathogens; successful disease development in nature is relatively rare and the majority of plant species are resistant to complete microbial species (Heath, 1991; Gurr and Rushton, 2005; Ingle et al., 2006; Hein et al., 2009). Host-parasite specificity can be distinguished between (a) nonhost resistance; when all members of a plant species are resistant to a pathogen, and (b) cultivar resistance within a susceptible host species as plants evolve the ability to successfully defend themselves against pathogen attack (Heath,

1981a,b). Nonhost resistance is the commonest form of disease resistance found in plants (Ingle et al., 2006), it relies on both constitutive structural or chemical barriers (such as the plant cell wall, waxy cuticle, actin microfilaments and antimicrobial compounds known as phytoanticipins (Kobayashi et al., 1992; Nürnberger and Lipka, 2005)) as well as inducible defences. These inducible defences are brought about by a plant's ability to recognise and respond to pathogen or microbial-associated molecular patterns (PAMPs or MAMPs).

The 'zig-zag' model by Jones and Dangl (2006) illustrates the interplay between pathogen effectors and the amplitude of the defence response that the plant is able to produce. This model has been further developed and tailored to describe the interaction of oomycete pathogens with their hosts and is reproduced in Figure 1.1 (Hein et al., 2009). The model describes the ability of plants to respond to PAMPs; termed pattern (or PAMP) triggered immunity (PTI). In order for successful colonisation, plant pathogens must be able to avoid detection (for example by evolution of non-recognised PAMPs) or have the ability to suppress the signalling events which lead to PTI. Successful pathogens achieve this through the deployment of effector proteins, known as effector triggered susceptibility (ETS). Pathogen effectors may then be recognised by host NB-LRR (nucleotide binding site plus leucine-rich repeat) proteins, therefore bringing about effector triggered immunity (ETI). ETI may then be avoided by the shedding of effectors by pathogens, or due to gain of additional effector proteins which suppress ETI. This host-pathogen arms-race then continues as selection favours plant NB-LRR alleles which can recognise new pathogen effector proteins.

1.3 PAMP Triggered Immunity (PTI)

PTI is achieved through recognition of PAMPs by pattern recognition receptors (PRRs) at the cell surface. Many are Leucine-rich repeat receptor kinases (LRR-RKs) which are akin to Toll-like receptors in mammals (Jones and Dangl, 2006; Boller and He, 2009). In addition to PAMP recognition, damage to host proteins induced by pathogens may also be detected by the host plant via recognition of damage associated molecular patterns (DAMPs) (Lotze et al., 2007). Examples of DAMPs include systemin in *Solanaceae* species and *At*PEP1 in *A. thaliana* (Pearce et al., 1991; Yamaguchi et al., 2006; Huffaker et al., 2006).

PRRs appear to specifically target conserved and necessary epitopes in PAMPs, for example the protein flagellin is the principle component of bacterial flagella and contains

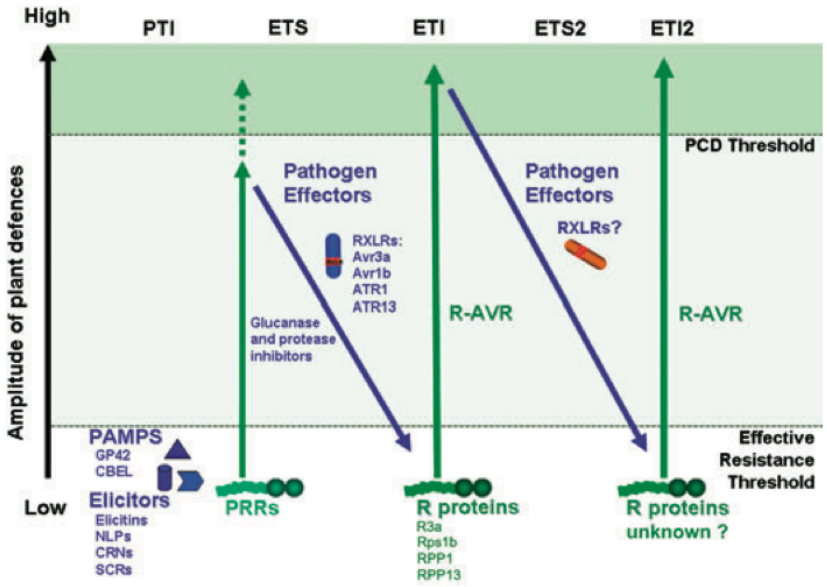


Figure 1.1: The Zig-Zag-Zig model for oomycete-plant interactions.
The Zig-Zag-Zig model for oomycete-plant interactions, including the characterised oomycete PAMPs and examples of oomycete effectors and the host resistance proteins which recognise them. PTI = PAMP-triggered immunity, ETI = effector triggered immunity, ETS = Effector triggered susceptibility, PCD = Programmed cell death, PRR = Pattern recognition receptor. From Hein et al. (2009).

a conserved stretch of 22 amino acids (flg22) which is recognised by the receptor FLS2 (Felix et al., 1999). An aspartic acid to valine substitution in flg22 from *P. syringae* pv. *tabaci* has been shown to abolish recognition but also render the microbe motionless and therefore reduce its virulence (Naito et al., 2008). However it is worth noting that the sequence of flg22 in some bacterial species such as *Agrobacterium tumefaciens* is highly divergent and not recognised by FLS2 yet these bacteria remain virulent (Felix et al., 1999). Another example of a plant PRR is EFR (EF-Tu receptor) which recognises the first 18 amino acids (termed elf18) from bacterial elongation factor Tu (EF-Tu) (Kunze et al., 2004).

Early oomycete PAMPs to be characterised were surface exposed β-Glucans from *Phytophthora megasperma* f.sp. *glycinea* (Pmg), which were found to elicit phytoalexin production in Soybean (Keen et al., 1983; Sharp et al., 1984). Recognition of oomycete glucans has since been found to be mediated by a soluble glucan-binding protein (GBP) in legumes (Umemoto et al., 1997; Fliegmann et al., 2004). Akin to recognition of flg22, an example of recognition of a conserved and necessary epitope in oomycetes has been identified. A peptide fragment (Pep-13), within the cell wall glycoprotein (GP42) from *Phytophthora sojae* has been found to elicit defense responses in parsley and potato. Mutation of Pep-13 has revealed that mutations which prevent recognition also prevent the

protein from functioning in its role as a transglutaminase (Nürnberger et al., 1994; Brunner et al., 2002). Other oomycete PAMPs and elicitors of plant immunity have been reviewed by Hein et al. (2009) and include cellulose-binding elicitor lectin (CBEL) (identified from *Phytophthora parasitica*) and the secreted protein INF1 from *Pi* which triggers PTI and cell death (Mateos et al., 1997; Kamoun et al., 1998).

The most extensively studied are those PRRs which recognise bacterial PAMPs and the associated downstream signalling. An overview of the current model for signalling in the FLS2 pathway is given in Figure 1.2 (Segonzac and Zipfel, 2011). Downstream signalling after detection by PRRs is mediated by leucine-rich repeat receptor-like kinases (LRR-RLKs). BAK1, a LRR-RLK belonging to the SERK subfamily (so called because of sequence homology to the carrot LRR-RK SERK (Hecht et al., 2001)), has been found to form a complex with FLS2 within minutes of stimulation by flagellin (Chinchilla et al., 2007; Heese et al., 2007; Roux et al., 2011). BAK1 has been found not to be necessary for flg22 binding, however *bak1* mutants show impaired flg22 responses. Early responses to elf18 are also compromised in *bak1* mutants, suggesting that BAK1 is involved in detection and downstream signalling mediated by EFR (Chinchilla et al., 2007; Heese et al., 2007). It has been suggested that reduced, rather than removal of PTI in the *bak1* mutant can be accounted for by redundancy in the SERK subfamily, and involvement of SERK4 in FLS2 and EFR-dependent signalling has been shown (Segonzac and Zipfel, 2011).

As shown in Figure 1.2, Ca^{2+} ion influx, causing a rise in cytosolic Ca^{2+} ion concentration is one of the early events in PTI signalling. The subsequent activation of calcium-dependent protein kinases (CDPKs) leads to transcriptional reprogramming in early PTI (Boudsocq et al., 2010). Mitogen-activated protein kinases (MAPK) are also triggered extremely rapidly after elicitation by PAMPs. Through the sequential transfer of phosphate groups; from a MAPK kinase kinase (MAPKKK) to a MAPK kinase (MAPKK) to a MAPK they are able to link upstream regulators to downstream targets. Their involvement in signalling between PAMP-receptors and responses has been indicated by treatment with flg22 resulting in activation of MPK3, MPK4 and MPK6 (Droillard et al., 2004). MPK3 and MPK6 have also been implicated in the regulation of camalexin production in response to the necrotrophic pathogen *Botrytis cinerea* (Ren et al., 2008).

The result of these signalling pathways are transcriptional changes, production of reactive oxygen species (ROS), hormone biosynthesis and callose deposition in the cell wall (Kunze et al., 2004; Navarro et al., 2004). ROS are generated via cell wall peroxidases, (for example PRX33 and PRX34 (Daudi et al., 2012)) and rapid generation of a ROS burst (consisting of superoxide, hydroxyl radicals, nitric oxide and hydrogen peroxide)

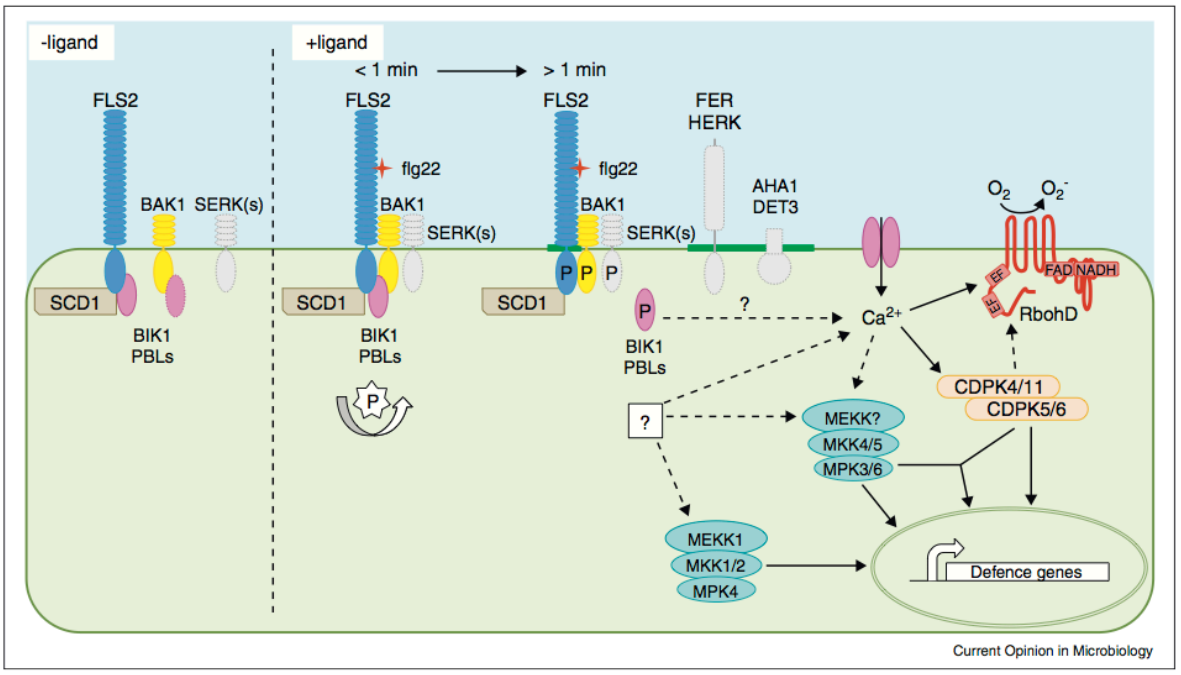


Figure 1.2: Model of the FLS2 perception and signalling pathway in *A. thaliana*. The current model for FLS2 signalling in *A. thaliana*. In response to flg22 binding, a complex form between FLS2, BAK1, BIK1 and other SERKs. Phosphorylation of these proteins occurs, followed by release of BIK1 from the complex and subsequent activation of CDPKs and MAPK cascades. Reproduced from Segonzac and Zipfel (2011).

is required for pathogen resistance (Lamb and Dixon, 1997; Bindschedler et al., 2006). Zipfel et al. (2004) found that broad transcriptional changes can be detected 30 minutes after plant treatment with flg22, including genes involved in antimicrobial action as well as signalling and transcriptional regulation (indicating feedback loops). Callose; a high molecular weight β-(1,3)-glucan polymer, is produced as a physical barrier to infection and a structural matrix in which anti microbial compounds may be deposited.

1.4 Pathogen Effector Proteins

Although there have been some rare examples of mutations in PAMPs which allow PTI avoidance (Pfund et al., 2004; Sun et al., 2006), successful pathogens will often deploy effectors to suppress the ability of plants to prevent pathogen proliferation by PTI. Effector proteins (sometimes termed elicitors) were defined by Kamoun (2006) as pathogen molecules that manipulate host cell structure and function thereby facilitating infection and/or triggering defence responses. However some effectors may also play structural roles such as in the extrahaustorial matrix which forms during oomycete and fungal infection (Jones and Dangl, 2006). Effectors are utilised by plant pathogens as a means

to suppress host immunity and enable successful infection, particularly in the case of biotrophs which need to avoid detection in oder to obtain nutrients from living plant tissue. They are utilised by a wide range of phytopathogens including bacterial effectors, which may be directly injected into the host cell via a type III or type IV secretion system and nematode effectors that are secreted through the stylet.

Numerous examples have been found of pathogen effectors which are able to target and suppress PTI; such as the fungal effector Ecp6 which prevents PTI triggered by the recognition of chitin by the Chitin Oligosaccharide Elicitor Binding Protein (CEBiP) receptor (Felix et al., 1993; de Jonge et al., 2010). Other examples of effectors targeting PTI are the *Pseudomonas syringae* effector AvrB (Shang et al., 2006) and the *Xanthomonas campestris* effector AvrAC which has been found to enhance virulence by targeting BIK1 and RIPK (two cytoplasmic RLKs which are involved in immune signalling) (Xu et al., 2008; Feng et al., 2012). The MAPK cascade in particular has been targeted by several *P. syringae* effectors, for example AvrPto and AvrPtoB which interrupt signalling upstream of MAPKKKs (He et al., 2006). The *P. syringae* effector HopAI1 also inhibits MAPKs, and achieves this by directly interacting with MPK3 and MPK6. The result is suppression of transcriptional activation of PTI genes, in addition to preventing reinforcement of cell wall defense such as by callose deposition (Zhang et al., 2007).

In addition, bacterial extracellular polysaccharides (EPSs) can be considered effectors as they meet the definition by Kamoun (2006). EPSs are able to suppress Ca^{2+} influx by calcium chelation, therefore preventing the downstream targets of CDPKs (Aslam et al., 2008). It is also likely that there are many uncharacterised examples of pathogen effector proteins which aid infection by suppression of PTI. For example, screens by Fabro et al. (2011) have shown that the presence of many candidate effectors from *Hyaloperonospora arabidopsidis* (*Hpa*) causes enhanced pathogen growth.

1.5 Effector Triggered Immunity (ETI)

Host plants have evolved the ability to recognise these effectors in a gene-for-gene interaction (Flor, 1971) whereby plant disease resistance loci (*R* genes) interact with corresponding pathogen effectors; also known as avirulence (*avr*) loci. Well characterised examples include the rice *R* gene *Pi-ta* which mediates resistance against isolates of the rice blast fungus *Magnaporthe grisea* expressing *AVR-Pita* (Bryan et al., 2000) and the *R3* resistance locus in potato which contains the *R* genes *R3a* and *R3b* and confers resistance to isolates of *Phytophthora infestans* which express *Avr3a* (Armstrong et al., 2005).

Most *R* genes encode polymorphic NB-LRR (nucleotide binding site plus leucine-rich repeat) protein products (Dangl and Jones, 2001), which show similarity to *Drosophila* and mammalian immune receptors. This similarity is thought to be an example of convergent evolution rather than shared ancestry (Ausubel, 2005). In *A. thaliana*, there are two *R* gene mediated signalling pathways, brought about by contrasting R protein types. The first of these are similar to *Drosophila* Toll and mammalian interleukin 1 transmembrane receptors, known as 'TIR' type. The second class have an N-terminal coiled coil (or 'CC') protein-protein interacting domain in place of the TIR domain, and are therefore known as CC-NB-LRRs (Aarts et al., 1998).

When an R-protein is in the presence of the corresponding avr protein, the result is rapid, localised, programmed cell death known as the hypersensitive response (HR) (Mur et al., 2008). Recognition may be through direct interaction of the gene products or, as it has been postulated, due to modification of a plant protein which is guarded by the R-protein (known as the guard hypothesis), therefore triggering a downstream resistance response (van der Biezen and Jones, 1998; McDowell and Woffenden, 2003). This state is known as effector-triggered immunity (ETI) and may drive natural selection upon pathogens to either acquire additional effectors to suppress ETI or to diversify and/or shed existing effectors.

Although brought about by different triggers, there are many similarities between PTI and ETI, for example they bring about overlapping transcriptional changes (Navarro et al., 2004) and both rely on MAPK cascades as an important regulatory component; silencing of MPK6 enhanced susceptibility of avirulent *Hpa* isolate Emwa1 in Col-0 plants as well as to virulent *Pst* and avirulent *Pst* due to the expression of *avrRpt2* (Menke et al., 2004). Interestingly however, this effect was not observed across all avirulent isolates tested (including *Hpa* isolate Noco2 with resistance mediated by *RPP7* (Holub et al., 1994) and *Pst* expressing *avrRpm1*), indicating that different pathways are involved in the downstream signalling of other resistance genes (Menke et al., 2004).

Phyto-pathogens can avoid ETI, either by shedding effectors that are recognised by the host plant, or by gaining effectors which are able to suppress ETI. An example of ETI suppression is shown by the ability of many type III effectors from *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) to suppress the hypersensitive response (HR) brought about by the avirulence genes *HopA1* and *avrRPM1* (Guo et al., 2009).

1.6 Plant Hormone involvement in Defence

Pathogens can be broadly categorised depending on how they obtain nutrition from their host plant; biotrophs (for example *Hpa* or *P. syringae*) which grow and reproduce in living plant tissue, necrotrophs that feed on dead plant cells (for example *B. cinerea*), or hemibiotrophs (for example *Pi*) which establish a biotrophic relationship with the host but subsequently cause host cell death as the infection proceeds (Parbery, 1996; Latijnhouwers et al., 2003; Solomon et al., 2003; Agrios, 2005). Plants need to use different strategies to defend themselves against biotrophic and necrotophic pathogens, for example programmed cell death may render a biotroph unable to obtain nutrients from its host but help the spread of a necrotrophic pathogen (Glazebrook, 2005).

Despite extensive study, the role of the plant hormones salicylic acid (SA), jasmonic acid (JA), ethylene (ET) and abscisic acid (ABA) in plant defense remains complex and is not fully understood. It is generally accepted that SA mediates defence to biotrophic pathogens whereas the JA/ET pathway is effective against necrotrophs (Thomma et al., 1998; Glazebrook, 2005).

1.6.1 Salicylic Acid

SA is a small phenolic compound which has a well documented role in downstream regulation of both PTI and ETI. SA was originally studied due to its importance for systemic acquired resistance (SAR); broad spectrum, systemic resistance which is effective against biotrophic pathogens (JA Ryals, 1996). It was observed that SA concentration increased after pathogen infection (Malamy et al., 1990; Rasmussen et al., 1991) and application of SA (and synthetic analogues) was shown to induce disease resistance (White, 1979; Lawton et al., 1996; Wang et al., 2007). The necessity of SA for the activation of defense responses has also been shown, plants lacking SA have been found to display increased susceptibility to pathogens. For example Col-0 *Arabidopsis* plants expressing bacterial salicylate hydroxylase *(nahG)* (facilitating the conversion of SA to catechol) showed enhanced susceptibility to tobacco mosaic virus and *P. syringae*, in addition to both virulent and avirulent isolates of *Hpa* (Noco and Wela respectively) (Delaney et al., 1994; JA Ryals, 1996; Tsuda et al., 2008).

The accumulation of SA results in synthesis of pathogenesis-related (PR) proteins (van Loon and Van Kammen, 1970; Bol et al., 1990; Ward et al., 1991). A role for PR proteins in resistance has been shown by their over-expression in transgenic tobacco, resulting in reduced susceptibility to oomycete pathogens (Loon, 1985; Alexander et al., 1993).

During attempts to characterise the signalling pathway leading to synthesis of PR proteins upon SA accumulation, the *npr1* (non-expresser of *PR* genes) mutant was identified, which showed a reduction in *PR-1* and *PR-5* expression following SA treatment (Cao et al., 1994).

The *A. thaliana* protein NPR1 (also called NIM1 (non-inducible immunity1) (Delaney et al., 1995)) is an important component of SA signalling. There are six members of the *A. thaliana* NPR1 family (Hermann et al., 2013). These have been found to show altered biochemical properties in the presence of SA, and to bind SA *in vitro*, therefore indicating that they are the receptors for SA (Maier et al., 2011; Wu et al., 2012). The NPR1 family members show different SA binding affinities, for example NPR1 only binds weakly to SA, whereas NPR4 binds with a high affinity (Fu et al., 2012).

NPR1 has been found to interact with the NIMIN (NIM1-interacting) proteins, four of which are encoded by the *A. thaliana* genome; NIMIN1, NIMIN1b, NIMIN2 and NIMIN3 (Weigel et al., 2001). Transgenic *A. thaliana* constitutively expressing NIMIN1 displayed repression of *PR* gene expression, therefore suggesting that NPR1 and NIMIN1 co-regulate SA responses (Weigel et al., 2005). All four *A. thaliana* NIMIN proteins contain a C-terminal EAR motif (Weigel et al., 2005) and it is likely that responses to SA are therefore transcriptionally repressed by recruitment of the co-repressor TPL (further discussed in section 1.10) (Arabidopsis Interactome Mapping Consortium, 2011). NPR1 has been shown to degrade in the presence of SA (mediated by NPR3 and NPR4), therefore relieving this repression and enabling the activation of SA responsive genes (Fu et al., 2012). It is important to note that there are also NPR1-independent SA signalling pathways, thus indicating multiple signalling pathways which lead to the full SA response (Shah et al., 2001).

1.6.2 Jasmonic Acid

The role of jasmonates in defense was first suggested by Farmer and Ryan (1992) who observed a role for jasmonates in triggering signalling in the wounding response. The importance of jasmonate in plant defense was further shown by the enhanced susceptibility to *Pythium mastophorum* observed in *A. thaliana* mutants which were unable to accumulate jasmonate (Vijayan et al., 1998). In addition, jasmonate insensitive mutants (*coi1*) were found to be more susceptible to *Alternaria brassicicola*, *Botrytis cinerea* and *Erwinia carotovora* (Thomma et al., 1998; Norman-Setterblad et al., 2000).

A model for the JA biosynthesis pathway was elucidated by the study of JA-deficient *A. thaliana* mutants (Turner et al., 2002). Downstream of JA, it is able to form the volatile

methyl ester form Methyl jasmonate (MeJA) as well as conjugating with isoleucine (Ile), forming JA-Ile. JA is conjugated to Ile by the enzyme JAR1, the importance of which is highlighted by the *A. thaliana jar1-1* mutant, which shows enhanced susceptibility to the pathogen *Pythium irregulare* (Staswick et al., 1998).

The mechanism by which transcriptional activation of JA responsive genes is brought about was elucidated in 2007. There are twelve JAZ (jasmonate ZIM-domain) proteins encoded in the *A. thaliana* genome, which were found to be negative regulators of the MYC transcription factors which transcriptionally activate jasmonate responses. The presence of JA-Ile was found to promote the physical interaction of JAZ proteins with COI1, causing them to be targeted by the SCF^{COI1} ubiquitin ligase for degradation by the 26S proteosome (Thines et al., 2007; Chini et al., 2007). *In planta* MeJA mediated JAZ degradation can be visualised in both *Nicotiana benthamiana* leaves transiently expressing JAZ3-GFP (Chini et al., 2007) and stably transformed *A. thaliana* roots expressing JAZ10-GFP (Shyu et al., 2012). The mechanism by which JAZ and COI1 perceive JA-Ile has been subsequently shown (Sheard et al., 2010). Understanding this mechanism for activation of jasmonate signalling allows greater understanding of how host defense is manipulated by pathogens; the virulence factor coronatine (COR) produced by phytopathogenic strains of *P. syringae* shows structural similarity to JA-Ile (Katsir et al., 2008) and therefore acts as a molecular mimic, although has been shown to be approximately a thousand fold more effective than JA-Ile at promoting the interaction between JAZ proteins and COI1 (Katsir et al., 2008).

The mechanism by which JAZ proteins bring about repression of JA-signalling in the absence of JA-Ile has recently been characterised. JAZ proteins have been found to recruit the co-repressor TOPLESS (TPL) and TPL-related proteins (TPRs) via interaction with Novel Interactor of JAZ (NINJA). This mechanism is illustrated in figure 1.3, reproduced with permission from Pauwels et al. (2010). It is worth noting that the same outcome may also be achieved by slightly different mechanisms; JAZ8 has been found to interact directly with TPL, without the need for the presence of the co-repressor NINJA (Shyu et al., 2012).

1.6.3 Hormone Crosstalk

1.6.3.1 Jasmonic Acid / Ethylene

The interplay between phytohormones makes it challenging to distinguish their individual effects. For example, the JA and ET pathways are often considered together, since it has

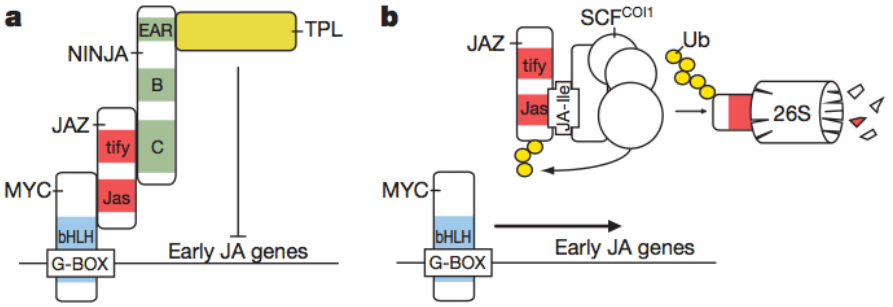


Figure 1.3: A model for the mechanism of JA signalling.
Mechanism of JA signalling (a) in the absence of jasmonates MYC transcription factors interact with JAZ proteins, which in turn recruit TPL via the co-repressor NINJA, leading to the repression of JA signalling. (b) In the presence of JA-Ile, JAZ proteins interact with SCF^{COI1} and are targeted for degradation by the 26S proteosome, relieving repression of early JA genes. Reproduced with permission from (Pauwels et al., 2010).

been found that both ET and JA pathways are required for defense gene activation in response to the pathogen *Erwinia carotovora* and the cell wall-degrading enzymes which it secretes (Norman-Setterblad et al., 2000). The protein ERF1 (ethylene response factor1) is a transcription factor which acts downstream of both JA and ET signalling and has been suggested to integrate these pathways (Lorenzo, 2002). Difficulty in separating responses may also be compounded by the difficulty in distinguishing between wounding responses and response to necrotrophic pathogens which cause damage and death to plant tissue. A role for ethylene is now thought to be modulation of disease resistance in plants; ethylene application has been observed to cause both enhanced disease resistance and enhanced susceptibility (reviewed in van Loon et al. (2006) and Adie et al. (2007)).

1.6.3.2 Salicylic Acid / Jasmonic Acid

It is well documented that the effects of SA and JA are antagonistic (Niki et al., 1998; Bostock, 2005; Beckers and Spoel, 2006), therefore allowing plants to tailor their defense response to meet the particular challenges poised by pathogens. It has been shown that when plants are treated with both SA and MeJA, expression of the JA responsive gene *PDF1.2* is repressed (Figure 1.4) (Koornneef and Pieterse, 2008). In addition, *A. thaliana* infection with *P. syringae* has been shown to make them more susceptible to the necrotrophic pathogen *Alternaria brassicicola* due to suppression of JA signalling (Spoel et al., 2007). There have also been examples of JA signalling antagonising SA defenses, and synergistic effects between the two phytohormones (Glazebrook, 2005).

There have been several studies trying to understand the mechanism behind SA / JA crosstalk. Application of SA has been shown to suppress the JA biosynthesis pathway

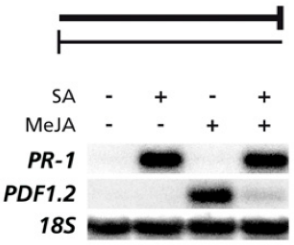


Figure 1.4: Antagonistic cross talk between SA and JA.
The expression of the SA responsive gene *PR-1* and the JA responsive gene *PDF1.2* show antagonism in the presence of both SA and MeJA. Reproduced from (Koornneef and Pieterse, 2008) with permission.

and it is through that this may suppress downstream JA responses (Pena-Cortes et al., 1993). A role for NPR1 in regulating SA / JA antagonism has also been discovered; *npr1-1 A. thaliana* mutants showed enhanced JA-responsive gene expression upon infection by *P. syringae* compared to wild type (Spoel et al., 2003). It has also been shown that ethylene plays a role in modulating this cross talk (Leon-Reyes et al., 2009).

1.6.3.3 Abscisic acid

ABA is known to play important roles in plant development and abiotic stresses such as salinity, drought and cold stress (Shinozaki et al., 2003). More recently, a role for ABA has been implicated in defense. Pre-treatment with ABA has been shown to increase susceptibility to *Phytophthora* species, specifically it was observed that incompatible *Pi* races grew and sporulated on potato tubers pre-treated with ABA (Henfling, 1980). This effect was also observed when soybean plants were pre-treated with ABA, allowing the growth of otherwise incompatible *P. sojae* (McDonald and Cahill, 1999) and *P. megasperma* (Ward et al., 1989) isolates. Moreover, pre-treatment with an ABA biosynthesis inhibitor (norflurazon) prevented the growth a normally compatible *P. sojae* isolate (McDonald and Cahill, 1999). In addition, the *A. thaliana* ABA-deficient mutant *aba1-1* displays reduced susceptibility to *Hpa* (Mohr and Cahill, 2003) and tomato mutants with reduced ABA levels displayed resistance to *B. cinerea*, which can be restored to a susceptible state by application of exogenous ABA (Audenaert et al., 2002).

ABA has also been shown to negatively regulate both SA and JA / ET mediated resistance to the necrotrophic fungus *Plectosphaerella cucumerina* (Audenaert et al., 2002; Sanchez-Vallet et al., 2012), and to antagonise SA-mediated induction of SAR at multiple steps in the signalling pathway (Yasuda et al., 2008). A summary of known crosstalk between SA and ABA during immunity is presented by Cao et al. (2011) and shown in Figure 1.5. There are some examples of SA and ABA acting synergistically, for example SA has been

found to act upstream of ABA during stomatal closure in response to pathogenic bacteria (Zeng and He, 2010).

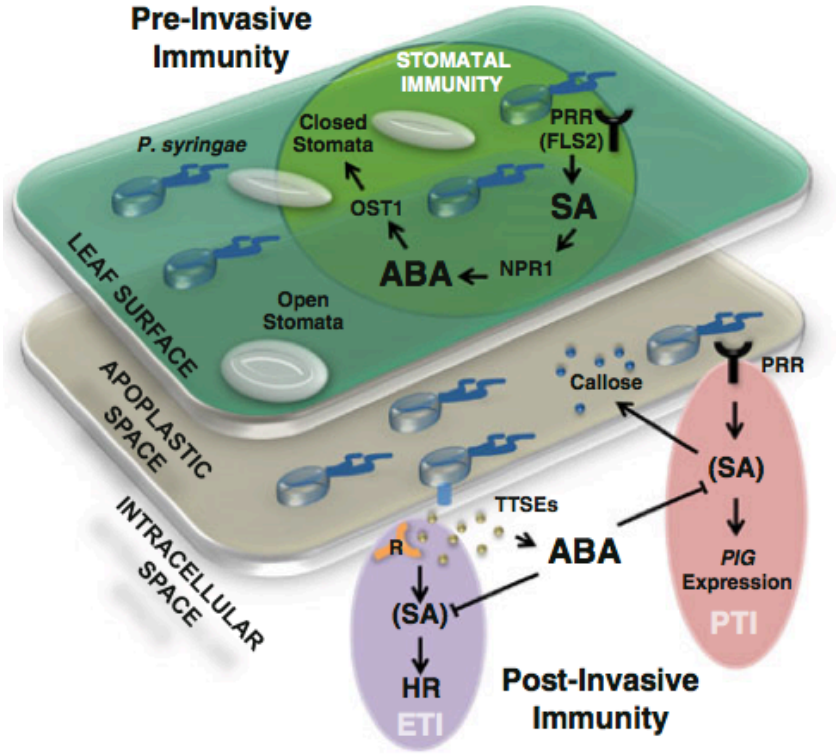


Figure 1.5: The involvement of ABA in immune responses.
The involvement of ABA in pre-and post-invasive immune responses and interplay with SA. TTSEs = Type three secreted effectors, PRR = Pattern recognition effector, PTI/ETI = PAMP/Effector triggered immunity, *PIG* = *PAMP-induced gene*. Reproduced from (Cao et al., 2011).

Antagonism between ABA and JA has also been observed; positive regulation of ABA signalling reduces expression of JA responsive genes (Anderson et al., 2004). Other hormones including auxin, gibberellin and cytokinins have also recently been implicated in defense and are reviewed in Robert-Seilaniantz et al. (2011).

1.6.4 Hormone manipulation by pathogens

Antagonism between hormone signalling pathways has been targeted by pathogens seeking to gain an advantage through manipulation of host defenses. One such example is *B. cinerea*, which produces an exopolysaccharide that manipulates the antagonistic effects between SA and JA signalling (El Oirdi et al., 2011). Another example is the previously mentioned bacterial virulence factor COR, produced by *P. syringae* which mimics JA-Ile

and results in supression of SA responses due to SA / JA crosstalk (Mitchell, 1982; Katsir et al., 2008). This is clearly an important virulence strategy as *A. thaliana jai1* mutants are insensitive to COR and show resistance to *Pst* DC3000 (Zhao et al., 2003). In addition to hormone mimics, there are many documented examples of pathogens producing phytohormones. These include *B. cinerea* and fungi in the genus *Fusarium* which produce ABA (Marumo et al., 1982; dorffling et al., 1984), and *Ralstonia solanacearum* which produces ET and a compound related to auxin (Valls et al., 2006).

Hormone pathways have also been targeted by pathogen effectors. The *p. syringae* effector protein avrPtoB modulates ABA antagonism to increase pathogen virulence (de Torres-Zabala et al., 2007). Additionally, the *X. campestris* effector AvrBs3 manipulates host transcription to cause the induction of auxin-responsive genes (Marois et al., 2002).

1.7 Oomycete effector proteins

In 2004, Allen et. al. identified ATR13 (*Arabidopsis thaliana* recognised 13) from *Hyaloperonospora arabidopsidis (Hpa)* (formerly classified as *Peronospora parasitica*) which is recognised by the protein encoded by the *R*-gene *RPP13* (Recognition of *Peronospora parasitica* 13) in *Arabidopsis thaliana*. Genetic mapping resulted in four oomycete effector genes being initially identified and cloned; ATR1 and ATR13 from *Hpa*, Avr1b-1 from *Phytophthora sojae* and Avr3a from *Pi*. All four of the initially cloned avirulence genes encode small, secreted hydrophilic proteins (Tyler, 2009). Alignment of the amino acid sequences encoded by the three genes ATR1, Avr3a and Avr1b-1 revealed a conserved motif. This motif, at the N-terminus of the protein (within 32 amino acids of the predicted signal peptide) consists of the consensus sequence RxLR (arginine, any, leucine, arginine). This sequence was further defined as RXLR-dEER (aspartate, glutamate, glutamate, arginine) and is shown in Figure 1.6 (Rehmany et al., 2005; Birch et al., 2006; Tyler, 2009).

Figure 1.6: The RxLR-DEER Motif

The RxLR-DEER motif identified by the alignment of ten oomycete effector proteins. The RXLR motif is displayed in red, acidic amino acids shown in green. Reproduced with permission from Rehmany et al. (2005)

It was noted that this motif was similar with the host-targeting signal (RxLxE/D/Q) (Whisson et al., 2007; Birch et al., 2008) which is conserved in numerous, dissimilar effector proteins from malaria parasites (*Plasmodium* spp.) and required for the export of effector proteins across both a pathogen derived membrane and an invaginated host membrane. It was therefore postulated that this motif may play a similar function in the delivery of oomycete effectors across the haustorial and plant cell membranes and has since been shown that RxLR-containing sequences from *Pi* can mediate host translocation of proteins from *Plasmodium* into an erythrocyte (Bhattacharjee et al., 2006). Necessity of the RxLR motif for translocation into host cells has since been shown in potato leaves by fusion of Avr3a to mRFP; without the RxLR-EER motif the protein was secreted into the haustoria and extra-haustorial matrix but not into the host cell (Whisson et al., 2007). Fur-

thermore, the RXLR-dEER domain from Avr1b was found to be sufficient for autonomous translocation of GFP into soybean root cells (Dou et al., 2008).

A motif similar to the RxLR motif has been identified in several candidate fungal effectors (Kale et al., 2010). The RxLR motif from oomycete effectors, in addition to this fungal RxLR-like motif was found to enable binding to phosphatidylinositol-3-phosphate (PI3P), a phospholipid which is abundant on the surface of plant cell membranes. This, combined with effector cell entry being inhibited by PI3P depletion, suggests a mechanism for effector translocation into host cells (Kale et al., 2010).

1.8 *Hyaloperonospora arabidopsidis* as a model oomycete pathogen

A model system that can be used to investigate the relationship between biotrophic oomycetes and their host plants is that of *Hyaloperonospora arabidopsidis* (formerly *Peronospora parasitica*), a naturally occurring pathogen which causes downy mildew of *Arabidopsis thaliana* (Slusarenko and Schlaich, 2003).

A. thaliana is a used as a model plant for the study of plant genetics due to many advantages including a short generation time, small size, small nuclear genome and the availability of the genome sequence (Arabidopsis Genome Initiative, 2000). It is therefore also useful as a model system for studying the basis of plant-pathogen interactions (Mauch-Mani and Slusarenko, 1993; Davis, 1998). This pathosystem was first described in detail in 1990 following the discovery of some *A. thaliana* plants naturally infected with *Hpa* (Koch and Slusarenko, 1990). It is hoped that by studying this system, results will then be able to be applied to related economically important crops, particularly close relatives of *Arabidopsis* within the *Brassicaceae*.

1.8.1 Lifecycle

The lifecycle of *Hpa* is illustrated by figure 1.7, reproduced from Coates and Beynon (2010). Asexual conidiospores land and germinate on the surface of an *A. thaliana* leaf (figure 1.7a), where they germinate and penetrate into the leaf using pressure formed by an appressorium (figure 1.7c), usually at the junction between two leaf epidermal cells. Feeding structures called haustoria then grow into these epidermal cells (figure 1.7d), however they are unable to penetrate the cell membrane and therefore the plasma membrane of the

plant cell is invaginated. Numerous hyphae then branch into intercellular spaces, forming numerous haustoria in mesophyll cells. Hyphal tips then develop into conidiophore primordials which emerge through stomata and expand into tree-shaped structures called sporangiophores that carry the asexual conidiospores (figure 1.7e) (Coates and Beynon, 2010).

Sexual reproduction occurs through the intertwining of hyphae, resulting in the formation of oospores which are able to overwinter in soil, one of the reasons why it is difficult to recover crop fields once infected by a plant-pathogenic oomycete.

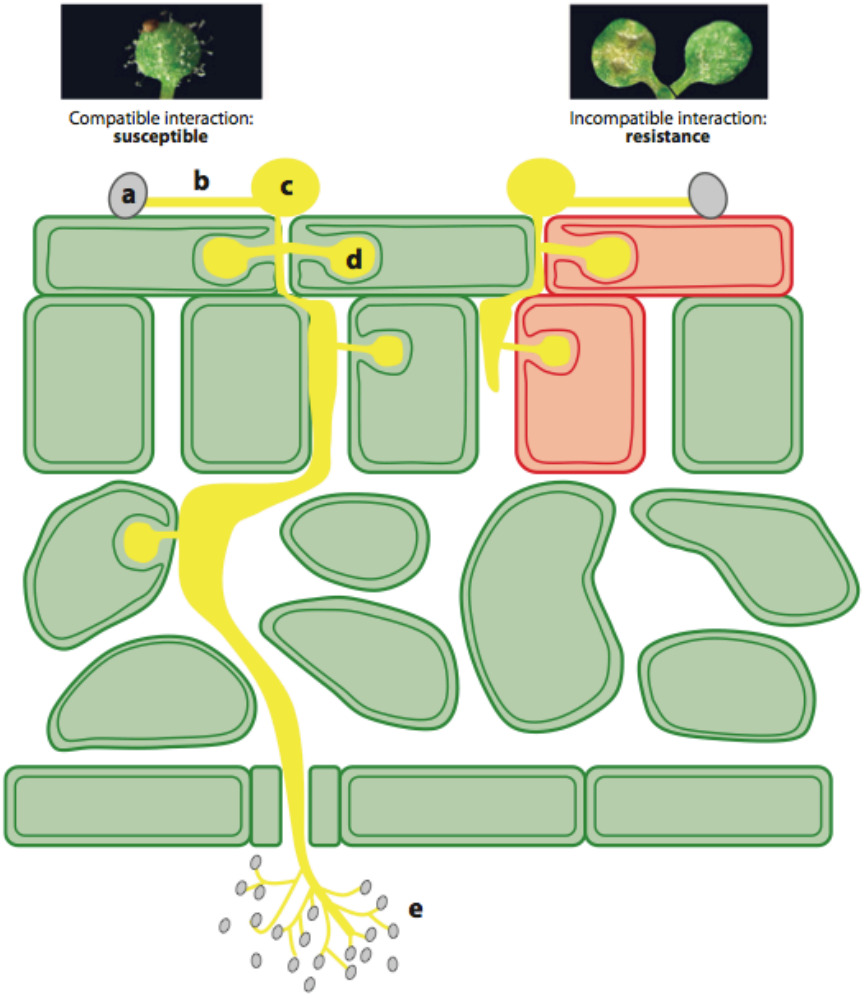


Figure 1.7: Compabible and Incompatible interactions of *Hyaloperonospora arabidopsidis* with *Arabidopsis thaliana*
The infection cycle of *Hyaloperonospora arabidopsidis*, showing a (L) compatible and (R) incompatible interacttion. (a) Asexual conidiospres land on the surface of the leaf, (b) germinate and (c) penetrate into the leaf via an appressorium. (d) Nutrients are obtained by haustoria are formed by invagination of the host cell membrane. Hyphae then grow throughout the leaf and (e) emerge through stomata, producing sporangiophores. Reproduced with permission from (Coates and Beynon, 2010).

1.8.2 Pathogenicity and effector proteins

It was noted that differential interactions existed between various isolates of *Hpa* and different *A. thaliana* accessions (Holub et al., 1994), therefore allowing for the cloning of resistance genes. These resistance genes have been termed *RPP* (Recognition of *Peronospora parasitica*), the first of which gene to be cloned was *RPP5*, cloned using map-based cloning from the *A. thaliana* ecotype *Lansberg erecta* (Parker et al., 1997). Subsequently, at least 27 *RPP* genes have been mapped in *A. thaliana* (Slusarenko and Schlaich, 2003; Coates and Beynon, 2010).

In 2007, the *Hpa* genome was made available (http://vmd.vbi.vt.edu/ download/index.php), thus allowing comparisons to be made with other, related plant pathogens as such as *Pi*. It has been found that pathogenicity genes such as those encoding host-targeted enzymes such as proteinases and pectin methyl esterases are reduced in *Hpa* (Baxter et al., 2010), possibly due to its biotrophic lifestyle. The availability of the genome sequence has also enabled putative effector proteins to be identified using the RxLR or RxLx motif as a guide. The draft genome sequence of *Hpa* (in addition to *Phytophthora sojae* and *Phytophthora ramorum*) was used to generate catalogs of RXLR effector genes, with a total of 149 candidate RxLR effectors being identified (Win et al., 2007). It was observed that the C-terminal of these effector candidates was undergoing positive selection, whereas the N-terminal remained conserved, concurrent with function of the N-terminus in secretion and translocation.

A total of 134 high-confidence effector candidates were subsequently identified from the genome of *Hpa* isolate Emoy2 (Baxter et al., 2010). Of these, a total of four avirulence genes have been cloned; ATR1, ATR13, ATR5, and ATR39 (Goritschnig et al., 2012). Attempts to characterise *Hpa* effectors have begun; the subcellular localisation has been elucidated using transient expression in *Nicotiana benthamiana* (Caillaud et al., 2011) and *A. thaliana* (Laura Lewis, personal communication). In addition, protein targets for effector proteins from *Hpa* and *P. syringae* have been identified by Mukhtar et al. (2011), who used yeast-two-hybrid to test for interactions between effectors and host (*A. thaliana*) proteins. They screened for interaction with 552 'immune proteins' such as N-terminal domains of NB-LRRs, cytoplasmic domains of RLKs and proteins known to be involved in immune signalling in addition to the ~8000 proteins used to generate the *A. thaliana* interactome (Arabidopsis Interactome Mapping Consortium, 2011). The result is a plant-pathogen immune network which can be mined for clues as to how these pathogens manipulate their host.

1.9 HaRxL21

It is possible to study the individual contributions to the infection process made by effectors, for example the membrane-associated effector HaRxL17 which confers enhanced susceptibility when expressed in *Arabidopsis* and localises to *Hpa* haustoria upon infection (Caillaud et al., 2012). Initial screens performed by Fabro et al. (2011) have also shown that multiple *Hpa* effector proteins suppress host plant immunity (as measured by infection with *Hpa*, *Pst*, callose deposition and ROS burst upon infection) when constitutively expressed in *Arabidopsis* plants. Notably, *Arabidopsis* plants expressing the candidate effector 'HaRxL21' display enhanced susceptibility to *H. arabidopsidis* isolate Noco2 and *P. syringae* ΔavrPto/ΔavrPtoB, as well as promoting growth of *Pst*-LUX in 8 of 12 *Arabidopsis* accessions tested (Fabro et al., 2011). The ability to compromise immunity in this way suggests that characterisation of HaRxL21 would offer insights into manipulation of the host immune system by this pathogen.

Alfano (2009) presents a "roadmap" for future research on plant pathogen effectors, with the final goal being to use the information gained to engineer plants with increased resistance to phytopathogens. The journey he describes is reproduced in figure 1.8. It begins with the identification of putative effectors from the pathogen genome sequence (such as has been done for RxLR effector candidates from the *Hpa* genome (Win et al., 2007)), followed by protein-protein interaction assays to identify targets in the host, as has been done by Mukhtar et al. (2011). The function of targets in the plant host can be elucidated, eventually leading to crop improvement (Alfano, 2009). This approach will be used for the study of HaRxL21 with the eventual aim of establishing important components of plant immune signalling.

HaRxL21 is a 45 kDa effector protein identified from the genome of *Hpa*. It contains a RLLR-DEER motif at the N terminus and an EAR motif (LMLTL) at the C terminus. Alleles of HaRxL21 have been found in *Hpa* isolates Cala2, Emco5, Emoy2, Hind2, Maks9 and Noks1. These alleles are generally conserved but SNPs can be observed and the Noks allele is truncated (therefore this allele does not contain an EAR domain). Using a matrix-two-hybrid approach (Mukhtar et al., 2011), HaRxL21 from Emoy2 was found to interact with four proteins; AT1G15750 (TPL, which has a role in auxin-dependent transcriptional repression (Osmont and Hardtke, 2008)), AT3G07780 (OBE1, which functions in protein binding), AT3G47620 (TCP14, a transcription factor) and AT5G55100 (Suppressor-of-White-Apricot; thought to be involved with RNA binding).

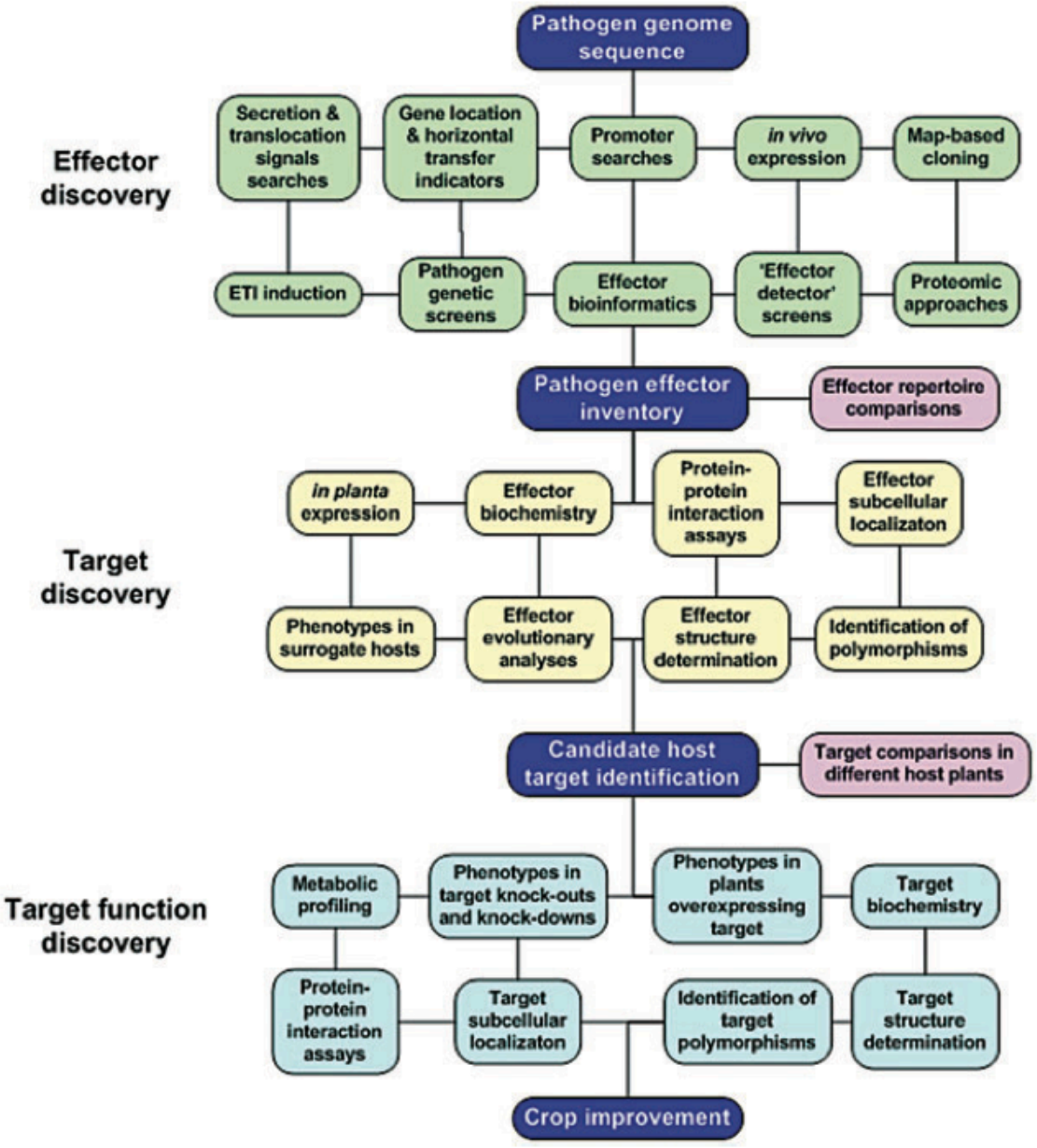


Figure 1.8: "Roadmap" for discovery of plant pathogen effectors and their targets. "Roadmap" for discovery of plant pathogen effectors and their targets from effector discovery within the pathogen genome sequence through host target identification and phenotype to eventual crop improvement. Reproduced from (Alfano, 2009).

1.10 EAR Motif containing proteins

Plants are sessile organisms and therefore developmental plasticity is especially important and one way by which this is achieved is through the action of the EAR motif. The Ethylene-responsive element binding factor-associated Amphiphilic Repression (EAR) motif was first identified in class II Ethylene resposnse factor (*ERF*) genes as the conserved sequence L/FDLNL/F(x)P (Ohta et al., 2001). Aux/IAA proteins were also found to contain a repression domain (LxLxL) (Tiwari et al., 2004) which showed similarity to the L/FDLNL/F(x)P motif in *ERF* genes and the 'EAR-like' sequence identified in the C terminal region of SUPERMAN (Hiratsu et al., 2002). It was subsequently shown that the DLELRL motif of SUPERMAN was necessary and sufficient for transcriptional repression in *Arabidopsis*, in particular the Leu residues within this motif (Hiratsu et al., 2004). Genome wide analysis of DLNxxP and LxLxL found that the EAR motif was detected in 10-25% of transcriptional repressors from a range of plant species (Kagale et al., 2010), and that these occurred with the highest frequency at the C-terminus of the protein. It was also found that the LxLxL motif occurs approximately three times more commonly than the DLNxxP motif (Kagale et al., 2010).

The mechanism by which EAR motifs bring about transcriptional repression is becoming clearer due to the discovery of EAR motifs mediating protein-protein interactions. For example IAA12 requires TOPLESS (TPL) for it's repressive activity; IAA12 interacts with TPL via it's EAR domain, and the CTLH domain of TPL (Szemenyei et al., 2008; Zeng et al., 2006). Another EAR-mediated interaction is the interaction of Novel Interactor of JAZ (NINJA) with TPL, connecting it to jasmonate signalling and allowing it to function as a transcriptional repressor. This interaction has been found to be abolished when Leu residues in the EAR motif were mutated to Ala (Pauwels et al., 2010). This mechanism of EAR-mediated protein-protein interaction has also been discovered in JAZ8, which, unlike other JAZ proteins does not require NINJA as an adaptor protein and recruits TPL directly via it's EAR motif (Shyu et al., 2012). It has been postulated that expression of target genes is controlled through chromatin modification of regulatory regions (Kagale and Rozwadowski, 2011), for example TPR1 (which shows 95% similarity to TPL at the amino acid level (Zhu et al., 2010) and 98% similarity at the CTLH domain which mediates interactions with the EAR motif (Kagale and Rozwadowski, 2011)) has been shown by coimmunoprecipitation to interact with *Arabidopsis* histone deacetylase 19 (HDA19) (Zhu et al., 2010), the action of which causes DNA to become more tightly packed and less accessible to transcription initiation complexes.

EAR domains have now been identified in many *Arabidopsis* proteins involved in stress

(such as RAP2.1 where the EAR domain is involved in regulation of response to cold stress (Dong and Liu, 2010)) and defence responses, for example at the C terminal region of NIMIN1 and related proteins, (which interact with NPR1 and modulate PR gene expression (Weigel et al., 2005)) and JAZ8 (which interacts with TPL and mediates jasmonate responses (Shyu et al., 2012)). The *Xanthomonas campestris* type III effector XopD has also been found to contain two tandemly repeated EAR motifs which are required for XopD-dependent virulence in tomato (Kim et al., 2008). XopD has been found to repress host defences by targeting the *Arabidopsis* transcription factor MYB30, however it has been shown that the EAR motifs are not sufficient for this process and suggested that they are involved in targeting a different component of the host defence response (Canonne et al., 2011).

1.11 Aims

The aim of this project is to elucidate the effect of HaRxL21 on the host plant, what it targets and its mode of action.

1. To determine the effect of HaRxL21 on the pathogenicity of a range of pathogens including both verifying the results observed by Fabro et al. (2011) and testing whether the presence of HaRxL21 confers a susceptibility advantage to the related oomycete pathogen *P. infestans*.
2. To verify putative interactions with TPL, TCP14, SWAP and OBE1 generated by the matrix-two hybrid (Mukhtar et al., 2011) using both Y2H and *in planta* methods.
3. To investigate transcriptional changes brought about by the presence of HaRxL21 in both the presence and absence of stimuli (pathogen infection and phytohormone treatment).
4. To determine the biochemical mechanisms by which HaRxL21 suppresses host immunity.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 General Laboratory Reagents

All general laboratory reagents were of analytic grade and were sourced from Sigma Aldrich (UK), Merck (Germany), Invitrogen (UK) or Scientific Laboratory Supplies Ltd. (UK), unless otherwise stated.

2.1.2 Molecular Biology Reagents

Oligonucleotides were supplied by Intitrogen or Integrated DNA Technologies (IDT; Scotland, UK). Polymerase chain reaction (PCR) reagents were contained in the BioMix™ 2x reaction mix (Bioline, UK); *Taq* (*Thermus aquaticus*) DNA polymerase, dNTPs and $MgCl_2$. QIAprep Spin Miniprep kit, QIAquick PCR Purification Kit, QIAquick Gel Extraction Kit, QIAGEN Plasmid Maxi Kit and RNeasy Plant Mini Kit were all obtained from Qiagen, UK. PCR purification plates were obtained from Millipore, UK. BP Clonase II and LR Clonase II enzymes were supplied by Invitrogen. The MessageAmp™ II aRNA kit was supplied by Ambion Europe Ltd (UK) and the Ovation® Pico WTA System V2 was supplied by NuGEN Technologies Inc. (U.S.A.). The Bioanalyzer 2100, RNA 6000 Nano Chip and reagents were obtained from Agilent Technologies, UK. Microarray slides were Complete Arabidopsis Transcriptome Microarray (CATMA) (NASC, UK), or 12 x 135k microarray slides (NimbleGen; Roche, Germany).

2.1.3 Electrophoresis Reagents

Agarose used was Type-I Ultrapure Agarose (Invitrogen) or Hi-Res Standard Agarose (AGTC Bioproducts). Gels were stained with Ethidium Bromide (Sigma Aldrich) or (2013 onwards) GelRed (Biotium Inc., U.S.A.). The DNA ladder used was Hyperladder I (Bioline).

2.1.4 Nucleic Acid Measurements

DNA and RNA concentration was measured using a NanoDrop ND-1000 (Thermo Scientific). Incorporation of Cy3 and Cy5 into cDNA for CATMA microarrays was also measured using this instrument.

2.1.5 Cell Density Measurements

Cell density measurements (OD_{600}) were taken using a Biochrom WPA CO8000 cell density meter (Biochrom Ltd., UK).

2.1.6 Vectors Used

1. **pDonr^{TM}zeo**; Gateway® entry vector. Also containing a Zeocin resistance gene for bacterial selection (Invitrogen^{TM}).

2. **pB2GW7**; Gateway® destination vector for expression of the gene of interest under the 35S promoter from Cauliflower mosaic virus. Also contains a Spectinomycin selectable marker for bacteria and Basta resistance gene for transgenic plant selection (Karimi et al., 2002).

3. **pGRAB**; Gateway® destination vector for expression of an untagged protein under a 35S promoter, also contains Kanamycin resistance gene for bacterial selection (Bos et al., 2010). Supplied by Petra Boevink.

4. **pDEST-AD (pAD)**; Gateway® destination vector for generation of Gal4 activation domain fusion to a protein of interest, for expression in yeast. Carbenicillin resistance gene, yeast selectable marker for growth on media lacking Tryptophan. (Dreze et al., 2010).

5. **pDEST-DB (pDB)**; Gateway® destination vector for generation of Gal4 DNA binding domain fusion to a protein of interest, for expression in yeast. Carbenicillin resistance gene for bacterial selection, yeast selectable marker for growth on media lacking Leucine. (Dreze et al., 2010).

6. **pK7WGF2**; Gateway® destination vector containing a GFP tag N-terminally fused to the protein encoded by the gene of interest, under the control of a 35S promoter. Also contains Spectinomycin resistance gene for bacterial selection and Kanamycin resistance for transgenic plant selection (Karimi et al., 2002).

7. **pK7FWG2**; Gateway® destination vector containing a GFP tag C-terminally fused to the protein encoded by the gene of interest, under the control of a 35S promoter. Also contains Spectinomycin resistance gene for bacterial selection and Kanamycin resistance for transgenic plant selection (Karimi et al., 2002).

8. **BIFP1**; Gateway® destination vector in which the N terminus of Clontech E-YFP is C-terminally fused to the protein encoded in the Gateway casette. Supplied by Francois Parcy (University Grenoble, France). Also contains Spectinomycin resistance gene for bacterial selection.

9. **BIFP2**; Gateway® destination vector in which the N terminus of Clontech E-YFP is N-terminally fused to the protein encoded in the Gateway casette. Supplied by Francois Parcy (University Grenoble, France). Also contains Spectinomycin resistance gene for bacterial selection.

10. **BIFP3**; Gateway® destination vector in which the C terminus of Clontech E-YFP is N-terminally fused to the protein encoded in the Gateway casette. Supplied by Francois Parcy (University Grenoble, France). Also contains Spectinomycin resistance gene for bacterial selection.

11. **BIFP4**; Gateway® destination vector in which the C terminus of Clontech E-YFP is C-terminally fused to the protein encoded in the Gateway casette. Supplied by Francois Parcy (University Grenoble, France). Also contains Spectinomycin resistance gene for bacterial selection.

12. **pEarleygate201**; Gateway® destination vector containing a HA tag N-terminally fused to the protein encoded by the gene of interest, under the control of a 35S promoter. Also contains Kanamycin resistance for bacterial selection and Basta resistance for transgenic plant selection (Earley et al., 2006).

13. **pEarleygate202**; Gateway® destination vector containing a Myc tag N-terminally fused to the protein encoded by the gene of interest, under the control of a 35S

promoter. Also contains Kanamycin resistance for bacterial selection and Basta resistance for transgenic plant selection (Earley et al., 2006).

14. **pEDV6**; Gateway® destination vector for delivery of inserted protein via the type III secretion system of *Pseudomonas syringae*, also contains a Gentamycin resistance gene for bacterial selection (Sohn et al., 2007).

15. **pBeaconRFP_GR**; Gateway® destination vector for transfection of protoplast suspensions, for expression of a gene of interest in frame with the rat glucocorticoid receptor (GR) protein. Also contains Ampicillin resistance for bacterial selection (Bargmann et al., 2013).

2.1.7 Plant Material

1. **Col-0 / Col-4**; *Arabidopsis thaliana* accession Columbia.

2. **ws-eds**; *Arabidopsis thaliana* accession Wassilewskija, enhanced disease susceptibility mutant (Parker et al., 1996).

3. **Col-0 GUS**; GUS protein cloned into pB2GW7 (see section 2.1.6 for vector details), transformed into Col-0 (section 2.2.2) and selected on BASTA until the fourth generation (transformation performed by Matthew Watson, University of Warwick).

4. **HaRxL21a/b/c**; HaRxL21 cloned into pB2GW7, transformed into Col-0 and selected on BASTA (Bayer CropScience, Wolfenbüttel, Germany) until the fourth generation. HaRxL21a, HaRxL21b and HaRxL21c differentiate between lines derived from independent transformations (transformation performed by Matthew Watson, University of Warwick).

5. **F1/3**; HaRxL14 cloned into pB2GW7, transformed into Col-0 and selected on BASTA until the fourth generation (transformation performed by Matthew Watson, University of Warwick).

6. **PR-1::GUS**; β-glucuronidase (GUS) protein fused to the PR1 promoter in a Col-0 background (Koornneef et al., 2008).

7. **HA::HaRxL21**; *Arabidopsis* Col-4 expressing HaRxL21 with an N-terminally fused HA tag (using Gateway vector Earleygate201), selected on Basta until homozygosity. HA::HaRxL21-4,-6 and -9 designate independent transformants (this study).

8. **HA::HaRxL21ΔEAR**; *Arabidopsis* Col-4 expressing HaRxL21 with the EAR domain truncated (HaRxL21ΔEAR) with an N-terminally fused HA tag (using Gate-

way vector Earleygate201), selected on BASTA until homozygosity. HA::HaRxL21ΔEAR-1,-8 and -9 designate independent transformants (this study).

9. **HA::HaRxL21 Cala2**; *Arabidopsis* Col-4 expressing the HaRxL21 allele from *Hpa* isolate Cala2 with an N-terminally fused HA tag (using Gateway vector Earleygate201), selected on BASTA until homozygosity. HA::HaRxL21 Cala-1,-6 and -9 designate independent transformants (this study).

10. **HA::GFP**; *Arabidopsis* Col-4 expressing GFP with an N-terminally fused HA tag (using Gateway vector Earleygate201), selected on BASTA until homozygosity (this study).

11. ***tpr1-tpl-tpr4*** *Arabidopsis* accession Col-0 with T-DNA insertions in the *tpr1*, *tpl* and *tpr4* genes. Provided by Zhu et al. (2010).

2.1.8 Microbial Strains

1. DH5α; Chemically competent *Escherichia coli* (*E. coli*) used for transformation. α-select Chemically Competent Cells, gold efficiency. Obtained from Bioline, UK.

   Genotype:
   F- *deo*R *end*A1 *rec*A1 *rel*A1 *gyr*A96 *hsd*R17(r_k^-, m_k^+) *sup*E44 *thi*-1 *pho*A Δ(*lac*ZYA *arg*F)U169 ϕ80*lac*ZΔM15λ-

2. *Agrobacterium tumefaciens* strain GV3101, used for stable transformation of *Arabidopsis thaliana* and transient transformation of *Nicotiana benthamiana* (Koncz and Schell, 1986).

3. HB101-pRK2013; *E. coli* Helper strain for triparental mating. Kan^{50} (Phadnis and Das, 1987).

4. DC3000; *P. syringae* pv. *tomato* DC3000 wild type. Rif^{100} (provided by Jonathan Jones, Sainsbury Laboratory).

5. DC3000 lux; *P. syringae* DC3000 with luciferase activity. Rif^{100} Kan^{25} (Fan et al., 2008).

6. *Pseudomonas fluorescens* (*PFO*) engineered to express the *hrp*/*hrc* cluster for effector delivery (Thomas et al., 2009).

7. *PFO*::AvrRPM1; *PFO* expressing the pVSP61 vector containing *avrRPM1* (Ritter and Dangl, 1996).

8. *PFO*::AvrRPS4; *PFO* expressing the pVSP61 vector containing *avrRPS4* (Ritter and Dangl, 1996).

2.1.9 Media and Buffers

LB growth media for *Escherichia coli*:
25 g of LB Broth, Miller (Fisher Scientific UK) per litre of MilliQ water, in 1.5% agar (VWR; UK).

Contains: 10 g / L Tryptone, 10 g / L NaCl and 5 g / L Yeast Extract.

Low salt LB growth media for *Escherichia coli*:
(This media is used when the antibiotic Zeocin is required.)
20 g of Low salt LB Granules, (Melford, UK) per litre of MilliQ water, in 1.5% agar.

Contains: 10 g / L Tryptone, 5 g / L NaCl and 5 g / L Yeast Extract

SOC media for *Escherichia coli* transformation:
Liquid medium from Invitrogen™ (catalogue number 15544-034).

Contains: 2% Tryptone, 0.5% Yeast Extract, 10 mM Sodium Chloride, 2.5 mM Potassium Chloride, 10 mM Magnesium Chloride, 10 mM Magnesium Sulphate and 20 mM Glucose.

YEB growth media for *Agrobacterium tumefaciens*:
5 g / L Beef Extract, 1 g / L Yeast extract, 5 g / L Peptone, 5 g / L Sucrose, 2 mM $MgSO_4$.

YEPD growth media for yeast:
10 g / L yeast extract, 20 g / L bacto-peptone, 50 ml / L of 40 % glucose, 15 ml / L of 65 mM adenine solution.

SC (Synthetic complete) selective growth media for yeast:
3.4 g / L yeast nitrogen base (lacking amino acids), 10 g / L ammonium sulfate, 2.6 g / L amino acid powder (lacking leucine, tryptophan, histidine and adenine). PH 5.9.

Amino acids added as required; 100 mM histidineHCl (light sensitive) 8 ml / L, 100 mM leucine 8 ml / L, 40 mM tryptophan (store at 4 °C) 8 ml / L, 65 mM adenine sulfate (sat-

urated) 15 ml / L.

KB growth media for *Pseudomonas syringae*:
20 g / L proteose peptone, 1.5 g / L K_2HPO_4, 1.5 g / L $MgSO_4$, 10 ml / L glycerol, pH 7.2.

MS media:
2.2 g Murashige Skoog nutrients (Murashige and Skoog, 1962) (Duchefa Biochemie, Haarlem, The Netherlands) per litre of MilliQ water, 1 % Sucrose and adjusted to pH 5.9 with Potassium Hydroxide, 0.7 % Plant agar (Duchefa Biochemie).

50 x TAE Buffer:
2M Tris base, 1M Acetic Acid, 50mM EDTA, pH 8-8.5 in MilliQ H_2O.

2.2 Methods

2.2.1 Plant growth

2.2.1.1 Soil

Seeds were planted onto 'Arabidopsis mix' at a density of approximately 50 seeds per unit of a p40 seed tray. They were covered with foil and vernalised at 4 °C for 2 days before being sealed with a transparent lid and insulation tape and placed in a growth room with short day conditions; 10 hours light, 20 °C, 60 % humidity. Ten day old seedlings were then transplanted into p24 seed trays (one seedling per unit) and sealed. The covers were removed after one week and the plants were grown for a further 3 weeks (or as necessary for the particular experiment) under the same conditions.

2.2.1.2 Plates

Seeds were aliquoted into 2 ml microfuge tubes and surface sterilised by the addition of 1 ml 70% ethanol for 10 minutes. The ethanol was removed and 50% bleach was added for 1 minute. The seeds were then rinsed with sterile MilliQ water until all the bleach was removed (approximately 5 washes) and stored in of 0.05% agarose at 4 °C for 2-3 days to vernalise. They were pipetted onto MS plates (Murashige and Skoog, 1962)

containing 1% sucrose, sealed with micropore tape and placed in a growth room with short day conditions; 10 hours light, 20 °C, 60 % humidity.

2.2.2 Plant transformation

A. thaliana plants of the Columbia ecotype were grown. Floral dipping was performed by Matthew Watson as described in Clough and Bent (1998). T0 seed was selected on either rockwool soaked in 1/2 MS and 5 μg / ml Basta or soil soaked in 5 μg / ml Basta. Resistant seedlings were transplanted and T1 seed generated which was then selected again by growth on Basta; lines which showed a 3:1 ratio of Basta resistance were selected and continued until the next generation. T2 seed was subsequently sown onto Basta-socked soil and lines which showed 100 % germination (and therefore resistance) were deemed homozygous.

2.2.3 PCR

PCR master mix was made as described in Table 2.1. PCR was performed using the GeneAmp® PCR System 9700 (Applied Biosystems), using the thermal cycling conditions described in Table 2.2.

Table 2.1: PCR Components used in all PCR reactions unless otherwise stated.

Component	Volume
BioMix® Red Master Mix	10 μl
Forward primer (10 μM)	1.5 μl
Reverse primer (10 μM)	1.5 μl
MilliQ H_2O	Up to a total of 20 μl

Table 2.2: Thermal Cycling Conditions used in all PCR reactions unless otherwise stated.

Step	Temperature	Time	Cycles
Polymerase Activation	95	5 min	1
Denaturation	95	30 sec	
Annealing	55	30 sec	28
Elongation	72	1 min 30 sec	
Final elongation	72	4 min	1
Cooling	15	Hold	

2.2.4 Cloning

2.2.4.1 PCR Amplification

To prepare inserts for Gateway® cloning, two-step PCR were used. Gene-specific primers were designed with AttB1/AttB2 mini sites, separated from the start or stop codon by a plant specific Kozak sequence (underlined below).

Forward: AAAAAGCAGGCTCACAAATG
Reverse: AGAAAGCTGGGTCACCGCCTCCGGATCA

PCR was performed using KOD hot start PCR master mix (Novagen) as described in Table 2.3 using the thermal cycling conditions described in table 2.4.

Table 2.3: PCR Components used for amplification with KOD Polymerase.

Component	Volume
KOD Master Mix	12.5 μl
Forward primer (10 μM)	0.75 μl
Reverse primer (10 μM)	0.75 μl
Template DNA	10 ng
MilliQ H_2O	Up to a total of 25 μl

Table 2.4: Thermal Cycling Conditions used in all PCR amplification with KOD polymerase.

Step	Temperature	Time	Cycles
Polymerase Activation	95	4 min	1
Denaturation	95	45 sec	
Annealing	55	30 sec	25
Elongation	68	1 min 30 sec	
Final elongation	68	7 min	1
Cooling	15	Hold	

PCR products were then cleaned up using a Millipore PCR cleanup plate and eluted in 30 μl water. One μl of this cleaned up PCR product was used as the template for a second round of PCR. This was performed using the same conditions, using full length AttB1/B2 primers as detailed below.

AttB1: GGGGACAAGTTTGTACAAAAAAGCAGGCT
AttB2: GGGGACCACTTTGTACAAGAAAGCTGGGT

PCR products were cleaned up by Gel extraction using a QIAquick Gel Extraction Kit, according to manufacturers instructions with a final elution volume of 30 μl.

2.2.4.2 BP/LR Reactions

All cloning here was performed using Gateway® cloning (Invitrogen™) according to the manufacturer's protocol.

2.2.4.3 *E. coli* transformation

Escherichia coli strain DH5α (Invitrogen™) was transformed by the addition of 1μl BP / LR reaction to 10 μl cells, followed by heat shock at 42 °C for 30 sec then incubation for 2 min on ice. Ninety μl of SOC medium containing no antibiotics was added and cells were incubated at 37 °C shaking for 1 hour. Transformed cells were then plated on LB agar containing the appropriate antibiotic selection, with overnight incubation at 37 °C.

2.2.4.4 Colony PCR

Colony PCR was used to determine correct insert size when cloning. Bacteria were picked and diluted in 50 μl of sterile water, followed by heating at 95 °C for 10 minutes for cell lysis. 4 μl was added to each PCR reaction and PCR was performed as described in section 2.2.3.

2.2.4.5 Plasmid preparation

Plasmid preparation was performed using a QIAprep Spin Miniprep kit (Qiagen) or a Thermo-scientific Miniprep kits, according to the manufacturer's protocol. The final elution volume used was 40 μl.

2.2.4.6 Sequencing

Plasmids were quantified and samples were prepared; 300-500 ng of DNA in 5 μl was combined with 5 μl primer at a concentration of 5 μM. Samples were sent to GATC Biotech for sequencing. When sequencing Gateway® entry clones in pDonr™zeo, M13 forward and reverse primers were used:

M13_Forward: GTAAAACGACGGCCAGTC
M13_Reverse: AACAGCTATGACCATG

2.2.5 *A. tumefaciens* mediated transient expression in *Nicotiana benthamiana*

2.2.5.1 Generation of *Agrobacterium tumefaciens* competent cells

A. tumefaciens strain GV3101 was inoculated into 10 ml of YEB medium containing Rif^{100} and $Gent^{30}$ and grown at 28 °C with 220 rpm shaking overnight. The following morning, the overight culture was inoculated into 200 ml of YEB medium containing Rif^{100} and $Gent^{30}$ and incubated at 28 °C (220 rpm shaking) for 4-6 hours, until an optical density of approximately 0.6-0.8 was reached. The flask was chilled on ice for 10 minutes with occasional shaking, followed by centrifugation of the culture at 2500 G at 4 °C for 20 minutes. The pellet was washed by resuspension in ice cold TE buffer (10 mM Tris/HCl, 1 mM EDTA pH 8.0). This centrifugation and washing step was then repeated. Cells were then centrifuged at 2500 G at 4 °C for 20 minutes and resuspended into ice cold YEB medium. 500 μl aliquots of cells were flash frozen in liquid nitrogen and stored at -80 °C.

2.2.5.2 *Agrobacterium tumefaciens* transformation

A. tumefaciens competent cells were thawed on ice. Approximately 1-2 μg of plasmid DNA was added to 100 μl of cells and mixed, before incubation on ice for 5 minutes. Cells were heat shocked by freezing at 5 minutes in liquid nitrogen followed by 5 minutes at 37 °C in a water bath. Cells were left on ice for 2 minutes followed by the addition of 900 μl of YEB medium containing no antibiotics. Cells were then incubated for 2-3 hours at 28 °C shaking, followed by centrifugation at 3000 rpm on a desktop microfuge for 30 sec. Approximately 900 μl of medium was removed and cells were resuspended in the remaining medium (approximately 10 μl). Cells were then plated on YEB containing appropriate antibiotics and incubated overnight at 28 °C.

2.2.5.3 Transient expression in *Nicotiana benthamiana*

A. tumefaciens strain GV3101 containing the clone of interest was grown overnight in 10 ml YEB medium at 28 °C 220 rpm shaking. *A. tumefaciens* containing a construct including the P19 silencing suppressor (Voinnet et al., 2003) was also grown. The following morning, cultures were centrifuged at 3000 G for 10 minutes. The supernatant was removed and cells were re-suspended in 10 ml infiltration buffer (10 mM MES, 10 mM $MgCl_2$ pH 5.7). The OD_{600} was then measured and adjusted, while mixing any constructs to be co-expressed. *A. tumefaciens* expressing P19 was added to each mixture at a final

OD_{600} of 0.6. Typically, a final OD_{600} of 0.05 was used when expressing constructs for confocal microscopy, and an OD_{600} of 0.6 was used for CO-IP or BIFC. 100 μM Acetosyringone was added to each cell suspension and incubated for 2-4 hours in the dark.

2.2.6 *Hyaloperonospora arabidopsidis* screens

2.2.6.1 Isolates used

Table 2.5: *Hpa.* isolates and *A. thaliana* accessions on which they are subcultured.

Isolate	*A. thaliana* accession for subculture	Growth on Col-0?
Emoy2	Tsu-1	No
Hiks1	Tsu-1	No
Noks1	Col-0	Yes
Maks9	Col-0	Yes

2.2.6.2 Subculture

Isolates were stored by freezing infected seedlings at -80 °C. Stored isolates were revived by suspension in sterile distilled water, drop infection onto 10 day old ws-eds plants and growth in a sealed propagator at 18 °C, with 10 hours light at 60 % humidity. After 7 days of growth, infected seedlings were harvested, suspended in sterile distilled water and vortexed. Spores were isolated from plant material by filtration through miracloth, counted using a haemocytometer and light microscope and adjusted to 30,000 spores per ml. Spores were then sprayed onto a compatible *A. thaliana* isolate as described in Table 2.5 and grown in a sealed propagator at 18 °C, with 10 hours light at 60 % humidity.

After revival from the freezer, isolates were subcultured twice in this way before use in pathogenicity screens.

2.2.6.3 Infection and Quantification

Plants were sown onto p40 seed trays at a density of approximately 25 seedlings per module. The modules around the periphery of the tray were sown with ws-eds and plant lines to be screened were randomised within the inner modules. Plants were grown under short day conditions; 10 hours light, 20 °C, 60 % humidity. Spores were harvested as described for subculture in section 2.2.6.2 and adjusted to 30,000 spores per ml (or 40,000 spores

per ml if to be grown on an incompatible *A. thaliana* accession). Spores were sprayed onto 14 day old *A. thaliana* seedlings (or 10 day old for incompatible accessions), propagators were sealed and grown at 18 °C, with 10 hours light at 60 % humidity. Counting was performed 4 days post infection using a dissecting microscope to determine the number of sporangiophores per seedling.

2.2.7 *Phytophthora infestans* screens

Effector proteins to be tested for *Pi* susceptibility effects were cloned into pGRAB (section 2.1.6) and transiently expressed in *Nb* as described in section 2.2.5.3, infiltrating at an OD_{600} of 0.1. P19 silencing suppressor was also infiltrated at an OD_{600} of 0.1, giving a total *A. tumefaciens* OD_{600} of 0.2.

Pi infections were performed at the James Hutton Institute, Dundee with Hazel McLellen. *Pi* tdTomato; *Pi* strain 88069 transformed to express tdt (tandom dimer tomato) flurescence protein (Saunders et al., 2012) was used. Sporangia were harvested from *Pi* cultures as described in (Bos et al., 2010) and inoculated onto *Nb* leaves as described in Whisson et al. (2007). Infected plants were grown at room temperature in sealed propagators. Seven days post infection, leaves were detached and imaged using an epifluorescent microscope to excite the tdtRFP. Lesion size was measured using Image J software (Schneider et al., 2012).

2.2.8 *Pseudomonas syringae* screens

2.2.8.1 Triparental Mating

Helper (*E. coli* strain HB101), donor (*E. coli* harbouring the EDV6 plasmid) and recipient (*P. syringae*) strains were streaked on agar plates (KB for *P. syringae* and LB for *E. coli*) containing appropriate antibiotics and grown overnight. The following morning, liquid cultures were inoculated in media containing appropriate antibiotics; 10 ml in KB at 28 °Cfor *P. syringae* and 5 ml in LB at 37 °C for *E. coli*.

After 7-9 hours of culture (or when the OD_{600} is between 1 and 2.5), cultures were spun down and resuspended in water. This wash step was then repeated and the OD_{600} of all cultures was adjusted to 0.2. Cultures were mixed into 1.5 ml microfuge tubes; 100 μl of donor, 100 μl of helper and 800 μl of recipient. Cultures were centrifuged at 12000 G for 30 seconds and resuspended in 0.1 ml of water, before plating as a drop on KB agar containing no antibiotics and overnight incubation at 28 °C. The following morning,

the drop of bacteria was suspended in 0.2 ml of sterile water, plated onto KB agar plates containing appropriate antibiotics and incubated at 28 °C for 2-3 days. Single colonies were re-streaked and colony PCR was performed to check insert sizes, as described in section 2.2.4.4.

2.2.8.2 *A. thaliana* infection and quantification

One day prior to infiltration, *P. syringae* strains were streaked out onto KB agar containing the appropriate antibiotics and incubated overnight at 28 °C. The following morning, bacteria were re-suspended from the plates by the addition of 4 ml sterile $MgCl_2$ and gentle mixing. The suspension was then spun down at 3500 G for 10 minutes and the supernatant discarded; this washing was then repeated to ensure all traces of media and antibiotics were removed. The OD_{600} was then measured and the suspensions were diluted to a final OD_{600} of 0.001 using sterile $MgCl_2$, corresponding to approximately $5x10^5$ CFU / ml. Leaves were then inoculated on the abaxial side using a sterile 1 ml syringe to infiltrate the whole leaf; four leaves on each of four plants were inoculated with each strain of bacteria, per timepoint. The plants were then placed in a sealed propagator in a growth chamber with 10 hours of light, at 22 °C and 60% humidity.

Samples were then collected immediately after infiltration, 24 h, 48 h and 72 hours post infection. Four leaves per plant were harvested and leaf disks were excised with a cork borer of 1 cm diameter. The tissue samples were placed in 2 ml microfuge tubes containing 200 μl of sterile $MgCl_2$. Two metal ball bearings were added and samples were ground in a mixer mill for 2 x 30 s at 25 Hz, until pieces of intact leaf tissue were no longer visible. After homegenisation a further 800 μl of $MgCl_2$ was added to each sample. Serial 1:10 dilutions in $MgCl_2$ were made and 8 μl spots were plated onto KB agar containing appropriate antibiotics. They were allow to dry, the plates were sealed and grown at 28 °C for 48 hours at which point colonies were counted.

2.2.9 Conductivity Assay

Bacterial strains were prepared and infiltrated at an OD_{600} of 0.2 using the standard procedure described in section 2.2.8.2. Twenty four leaves across six plants were inoculated with each bacterial strain and twenty four leaves were also inoculated with sterile $MgCl_2$. Immediately after infiltration, 8 leaf discs (6 mm diameter) were cut (1 per leaf) and floated in 5 ml sterile water in a 6 well plate. Three replicates were prepared in this way per bacterial strain. After 30 minutes, the water was changed and the discs were floated

in 5 ml of water. The conductivity in each well was then measured at hourly time points.

2.2.10 *Botrytis cinerea* screens

2.2.10.1 *B. cinerea* subculture

Botrytis was sub-cultured at 10-14 days when the spores were clearly visible. A can of apricot halves was surface sterilised then the contents rinsed three times with sterile water to remove the sugar-water from the tin. The apricot halves were then air dried in a Category 2 hood for approximately 5 minutes. Each apricot half was placed in a deep petri dish and inoculated using a sterile pipette tip by grazing the surface of the existing botrytis culture then making contact with the apricot half. Each petri dish was then sealed with micropore tape and stored at 25 °C in the dark.

2.2.10.2 *B. cinerea* infection and quantification

Propagators were surface sterilised and filled with 800 ml of 0.8% bacterial agar. Plants were used at six weeks of age, one leaf per plant was detached and placed on the surface of the agar, with a total of 24 leaves per line.

A Botrytis culture was selected which was 14 days old and appeared to be showing even sporulation. Spores were then suspended in sterile distilled water and filtered through glass wool to remove any mycelium in the suspension. The spores were then counted using a haemocytometer and adjusted to 100,000 spores per ml in 50% sterile grape juice. A 10 μl drop of this spore suspension was then inoculated onto the adaxial surface of each leaf. A control leaf from each line was inoculated with 50% grape juice. The propagator was stored in a Sanyo cabinet with a constant temperature (22 °C), 90% humidity and 16h of light.

Photos of the propagators were taken 24 h, 48 h, 72 h and 96 h post-infection. ImageJ (Schneider et al., 2012) software was used to record lesion area on all of the leaves, using the ruler in the propagator to ensure the measurements were to scale.

2.2.11 Biochemical Techniques

2.2.11.1 Protein Extraction and Quantification

Tissue was harvested from plants and flash frozen in liquid nitrogen. In the case of protein transiently expressed in *Nicotiana benthamiana*, two leaf discs of 1.5 cm diameter were used per sample. Tissue was ground using the mixer mill for 2 x 30 seconds at 25 hz. A crude protein extract was performed by the addition of 400 μl of LDS sample buffer (NuPAGE® LDS Sample Buffer, Life Technologies) followed by 20 minutes shaking at 70 °C. Samples were quantified using Bradford reagent and comparison to a standard curve of Bovine Serum Albumin (BSA), then stored at -20 °C.

2.2.11.2 Western Blotting

Gel wells were washed with running buffer (table 2.6). Protein concentrations were adjusted and 10 μg loaded per well, 4 μl Spectra™ Multicolor Broad Range Protein Ladder (Thermo Scientific) was also loaded. Gels were run using MOPS running buffer (NuPAGE® MOPS SDS Running Buffer, Life Technologies) for 15 minutes at 90 V followed by 1 hour and 15 minutes at 120 V. The gel was removed from the gel tank and incubated in transfer buffer while the blot was prepared. The membrane was activated by rinsing in methanol for 30 seconds followed by rinsing in de-ionised water for 30 seconds then incubation in transfer buffer. A 'sandwich' of filter paper-membrane-gel-filter paper was assembled, supported by sponges pre-soaked in transfer buffer. Wet blotting was performed using a XCell II™ Blot Module (Life Technologies) at a constant 25 V for 2 hours, using ice water to keep the temperature of the blot cool.

α-HA:
For detection of HA tagged proteins the membrane was blocked overnight at 4 °C in 1% BSA in TBS 0.1% Tween20. The α-HA primary antibody (Anti-HA High Affinity Rat Monoclonal, Roche Applied Science) (diluted 1:2000 in TBS 0.1% Tween20 1% BSA) was applied for 2 hours. The membrane was washed for 3 x 10 minutes in TBS 0.1% T then the secondary α-rat antibody was applied for 2 hours. The membrane was washed for 3 x 10 minutes in TBS 0.1% Tween20 then was imaged.

Alternatively, an Anti-HA-HRP antibody (Miltenyi Biotech) was used. This was diluted 1:5000 in TBS 0.1% Tween20, 1% BSA and no secondary antibody was necessary.

α-GFP:
For detection of GFP tagged proteins the membrane was blocked overnight at 4 °C in

1% BSA in PBS 0.1% Tween20. The α-GFP-HRP antibody (Miltenyi Biotech) (diluted 1:5000 in PBS 0.1% Tween20 1% BSA) was applied for 2 hours. The membrane was washed for 3 x 10 minutes in PBS 0.1% Tween20 then was imaged.

α-Myc:
For detection of Myc tagged proteins the membrane was blocked overnight at 4 °C in 5% skim milk in PBS 0.05% Tween20. The membrane was washed for 3 x 10 minutes in PBS 0.05% Tween20 then the α-Myc primary antibody (diluted 1:2000 in PBS 0.05% Tween20) was applied for 2 hours. The membrane was washed for 3 x 10 minutes in PBS 0.05% Tween20 then the secondary α-rabbit antibody was applied for 2 hours. The membrane was washed for 3 x 10 minutes in PBS 0.1% Tween20 followed by imaging.

Alternatively, an Anti-Myc-HRP antibody (Miltenyi Biotech) was used. This was diluted 1:7500 in PBS 0.1% Tween20, 1% BSA and no secondary antibody was necessary.

Table 2.6: Western Blot Buffers

Buffer	Components per litre
SDS Sample Buffer	50 mM Tris-HCl pH 6.8 4 ml 100% glycerol 12.5 mM EDTA 1% β-mercaptoethanol 0.02 % bromophenol Blue 2% SDS
Transfer Buffer	2.4 g Tris 11.3 g Glycine 20 % Methanol

2.2.11.3 CO-IP

Nuclear Extraction of Protein

Protein was expressed by *A. tumefaciens* mediated transient expression in *Nb* as described in section 2.2.5.3. To ensure that enough tissue was provided, whole leaves were infiltrated. Tissue was harvested 2 days post-infiltration, flash frozen in liquid Nitrogen and stored at -80 °C.

Frozen leaves were ground in liquid nitrogen using a chilled pestle and mortar. After complete homogenisation, 6 ml of Nuclear isolation buffer (Sigma Aldrich) was added per gram of tissue. Tissue was then filtered through a filter mesh with 100 μm pores and centrifuged for 10 minutes at 1260 G. The pellet was resuspended in 2 ml buffer; 10ml

Nuclear isolation buffer, 1 Protease inhibitor tablet (Roche complete ULTRA Tablets, Mini, EDTA-free), Triton X-100 to a final concentration of 0.3% and centrifuged for 5 minutes at 12000 G. This washing step was then repeated 5 times until the pellet was pale green / white.

Co-immunoprecipitation

Nuclear enrichment was performed as described in section 2.2.11.3. Samples were then resuspended in 1 ml lysis buffer (10 ml lysis buffer as supplied with μMACS HA Isolation Kit (Miltenyi Biotec) plus 1 Protease inhibitor tablet (Roche)) and sonicated for 5 seconds, followed by 30 seconds on ice. This was repeated 5 times followed by centrifugation at 12000 G for 10 minutes. A sample of 100 μl from the supernatant was added to 900 μl of 100% ice cold acetone, forming the 'IP-input' sample. The remaining supernatant was transferred to a clean 2 ml microfuge tube and made up to 2 ml with lysis buffer.

50 μl of μMACS Anti-HA MicroBeads (μMACS HA Isolation Kit, Miltenyi Biotec) were mixed with 1 ml of lysate, inverted and incubated on ice for 45-60 minutes. A μColumn (Miltenyi Biotec Cat. No. 130-042-701) was placed on a magnetic stand (MACS MultiStand, Miltenyi Biotec) and washed with 200 μl lysis buffer. For each sample (lysate/beads), a 333 μl aliquot was loaded onto a column and this was repeated until the entire sample was loaded. The column was then washed with 400 μl lysis buffer, followed by 300 μl and twice with 200 μl. Columns were then washed with 100 μl 20 mM Tris HCl pH 7.5.

Elution buffer (SDS sample buffer supplied in μMACS HA Isolation Kit) was preheated to 95 °C, 20 μl was applied to columns and incubated for 5 minutes. Any droplets were removed from the tip of the column and 50 μl of preheated elution buffer was applied to each column, the flowthrough was then collected in a 1.5 ml microfuge tube. Samples were stored at -20 °C and western blots were performed as described in section 2.2.11.2.

2.2.11.4 TPL stability in the presence of HaRxL21

Proteins were expressed by *A. tumefaciens* mediated transient transformation of *N. benthamiana* as described in 2.2.5.3. *A. tumefaciens* harbouring a construct expressing Myc::TPL was infiltrated at an OD_{600} of 0.16 in all samples. *A. tumefaciens* harbouring a construct expressing HA::HaRxL21 or HA::HaRxL21ΔEAR was infiltrated at increasing OD_{600}; 0.01, 0.04, 0.08 and 0.16. The total OD_{600} was made up to 0.16 by *A. tumefaciens* harbouring a construct expressing HA::mRFP. Protein was extracted as described in section

2.2.11.1 and western blots were performed as described in section 2.2.11.2.

2.2.12 Confocal Microscopy

All confocal microscopy (unless otherwise described) was performed using a Zeiss Laser Scanning Microscope (LSM) 710 (Carl Zeiss Ltd; Cambridge, UK). Images were processed (including the addition of scale bars) using the Zeiss 2011 software (Zeiss). Typically the 40x objective lens was used.

2.2.13 BIFC

Protein was expressed in *Nb* as described in section 2.2.5.3. Constructs were co-expressed so that both the C and N terminus of E-YFP was present in the leaf; BIFP1 or 2 with BIFP3 or 4 (section 2.1.6). Two days post infiltration and prior to imaging, leaves were infiltrated with DAPI (Kapuscinski, 1995) at a concentration of 1 μg / ml. Leaf discs were then taken and imaged as described in section 2.2.12.

2.2.14 Yeast-Two Hybrid (Y2H)

Y2H screening was performed as described in Dreze et al. (2010) and (Mukhtar et al., 2011). Screens were performed in both directions, with each protein of interest fused to the DNA binding domain (pDEST-DB) and to the transcriptional activation domain (pDEST-AD) (Gateway^{TM} vectors as described in section 2.1.6).

2.2.15 CATMA Microarrays

2.2.15.1 *B. cinerea* infection for Catma microarray samples

B. cinerea was sub-cultured and plant lines inoculated as described in section 2.2.10.1 and 2.2.10.2 with the exception that five, equally spaced 7 μl drops were inoculated onto each leaf. Two samples for each line tested were taken at 0 h (un-inoculated), 2 h, 17 h and 22 h post-infection. Each sample consisted of 3 leaves pooled in a microfuge tube and flash frozen in liquid nitrogen.

2.2.15.2 RNA Extraction

Samples were stored at -80 °C and transfered to liquid Nitrogen. To each 2 ml tube containing frozen plant material a ball bearing (baked overnight at 180 °C to remove RNases) was added. Material was ground using a mixer mill for 30 seconds at 25 Hz.

In a fume hood, 1 ml of Trizol was added to each tube of ground plant material, vortexed and left at room temperature for 5 minutes. 200 μl chloroform was added to each tube which was vortexed and left for a further 5 minutes at room temperature. Samples were centrifuged at 14,000 rpm for 15 minutes at 4 °C and the top phase was transfered to a clean 1.5 ml microfuge tube. An equal volume of 70% ethanol was added to each sample, which was then transfered to a Qiagen RNeasy mini spin column. The manufacturers protocol was then used for RNA clean-up, after which samples were quantified by Nanodrop and stored at -80 °C. The integrity of the RNA was assessed using the Agilent Bioanalyser System (Bioanalyzer RNA 6000 Nano kit).

2.2.15.3 Amplification and Labelling

RNA was amplified using the MessageAmp^{TM} II aRNA Kit (Life Technologies). It was then labelled with Cy3 or Cy5 dCTP (GE Healthcare PA53021[Cy3-dCTP] PA55021 [Cy5-dCTP]) by incubation at 42 °C in the dark for 2.5 hours. Each reaction was halted by the addition of 2 μl of 2.5 M NaOH and incubated for 15 minutes at 37 °C, followed by the addition of 10 μl of 2M MOPS buffer and storage on ice. Samples were then purified using the Qiagen QiaQuick PCR Purification kit (manufacturers protocol). The concentration of each sample of labelled cDNAwas then measured using the Nanodrop spectrophotometer at 532 nm (Cy3) or 635 nm (Cy5).

2.2.15.4 Experimental Design

A loop design was used, ensuring that every sample was represented by both Cy3 and Cy5, comparable to every other sample and that technical replicates were included.

2.2.15.5 Array Hybridisation, Washing and Scanning

CATMA arrays were incubated for 1 hour at 42 °C in pre-warmed pre-hybridisation buffer:

Array slides were then washed in sterile MilliQ H_2O in a hybridisation rack followed by washing with isopropanol and drying by centrifugation. Each of the slides was then

Table 2.7: Microarray experimental design

	Cy3			Cy5	
Array	Line	time	Biorep	line	time	Biorep
1	HaRxL211	22 h	Rep b	HaRxL21a	0 h	Rep b
2	HaRxL21b	0 h	Rep b	Col-0	22 h	Rep a
3	HaRxL21a	22 h	Rep a	HaRxL21b	0 h	Rep b
4	HaRxL21a	0 h	Rep a	Col-0	22 h	Rep b
5	Col-0	22 h	Rep b	HaRxL21b	0 h	Rep a
6	Col-0	0 h	Rep b	HaRxL21a	22 h	Rep a
7	HaRxL21b	22 h	Rep a	HaRxL21b	22 h	Rep b
8	Col-0	22 h	Rep b	Col-0	0 h	Rep b
9	Col-0	0 h	Rep b	Col-0	0 h	Rep a
10	Col-0	0 h	Rep a	HaRxL21b	22 h	Rep b
11	Col-0	0 h	Rep a	Col-0	22 h	Rep a
12	HaRxL21b	22 h	Rep a	Col-0	0 h	Rep b
13	HaRxL21b	0 h	Rep b	HaRxL21b	0 h	Rep a
14	Col-0	22 h	Rep a	Col-0	22 h	Rep b
15	HaRxL21a	0 h	Rep b	HaRxL21a	0 h	Rep a
16	HaRxL21b	22 h	Rep b	HaRxL21a	0 h	Rep a
17	HaRxL21a	22 h	Rep a	HaRxL21a	22 h	Rep b
18	HaRxL21a	22 h	Rep b	Col-0	0 h	Rep a
19	HaRxL21b	0 h	Rep a	HaRxL21a	22 h	Rep b
20	HaRxL21b	22 h	Rep b	HaRxL21b	0 h	Rep b
21	HaRxL21b	0 h	Rep a	HaRxL21b	22 h	Rep a
22	HaRxL21a	0 h	Rep a	HaRxL21a	22 h	Rep a
23	HaRxL21a	0 h	Rep b	HaRxL21b	22 h	Rep a
24	Col-0	22 h	Rep a	HaRxL21a	0 h	Rep b

placed in a hybridisation chamber (Corning Cat. Number 2251).

Forty pmol of Cy3 labelled cDNA was combined with Cy5 labelled cDNA to be applied to the same array slide (according to the experimental design) and freeze dried. They were re-suspended in 50 μl of Hybridisation buffer (table 2.9), incubated at 95 °C for 5 minutes and centrifuged at 13000 rpm for 1 minute before applying to a CATMA array, applying a coverslip (Sigma Aldrich Cat. Number Z370274-100) and the cover of the hybridisation chamber. They were then incubated at 42 °C in the dark for 16-20 hours. Arrays were washed in saline-sodium citrate (SSC) buffer, immersed in isopropanol and dried by centrifugation.

Arrays were stored in the dark until scanning with a 428 Affymetrix scanner at 532 nm (Cy3) and 635 nm (Cy5).

Table 2.8: Pre-hybridisation Buffer

Components	Volume
Bovine Serum Albumin (Sigma A9418)	1.2 g
20x SSC	30 ml
14 % SDS	860 μl
Sterile MilliQ H_2O	90 ml

Table 2.9: Hybridisation Buffer

Components	Volume
Formamide	12.5 μl
20x SSC	12.5 μl
14 % SDS	0.35 μl
4μg/μl Yeast tRNA (Invitrogen)	6.25 μl
Sterile MilliQ H_2O	18.4 μl

2.2.15.6 Data Analysis

ImaGene 9.0 (BioDiscovery) was used to produce intensity readings from images of scanned microarray slides. The MicroArray Analysis of Variance (MAANOVA) package (Wu et al., 2003, 2008), in the statistical package 'R' (version 2.14) (http://www.r-project.org/) was used for data normalisation and statistical analysis. The data were normalised using locally weighted scatter-plot smoothing (LOWESS); both normalisation within arrays (rLOWESS) and normalisation within arrays (mgLOWESS). Data quality was then assessed visually by box plots before and after normalisation, in addition to ratio-intensity (RI) plots, grid checks and histograms. T-tests were performed within the MAANOVA package (Wu et al., 2003, 2008). The BiNGO (Biological Network Gene Ontology) plugin for Cytoscape (Maere et al., 2005) was used to identify over-represented Gene Ontology terms in the data.

2.2.16 NimbleGen Microarrays

2.2.16.1 Sample Preparation

Seedlings were grown on MS plates (as described in section 2.2.1.2 with the exception that seedlings were not transplanted until 21 days of age). Seedlings were then sprayed with either water or 100 μM SA. RNA was extracted as described in section 2.2.15.2. The integrity of the RNA was assessed using the Agilent Bioanalyser System (Bioanalyzer RNA 6000 Nano kit).

2.2.16.2 RT-PCR

RNA was reverse transcribed into cDNA using a QuantiTect kit (Qiagen) according to manufacturers instructions. 1.5 μl of the reverse transcription (RT) reaction product was then used for subsequent PCR (as described in section 2.2.3 but using a 30 second elongation time) to determine marker gene expression levels.

RT-PCR Primers used:

***Act2* F**: ACCTTGCTGGACGTGACCTTACTGAT
***Act2* R**: GTTGTCTCGTGGATTCCAGCAGCTT
***UBQ5* F**: AAGAAGACTTACACCAAGCCGAAG
***UBQ5* R**: ACAGCGAGCTTAACCTTCTTATGC
***PR1* F**: ACACGTGCAATGGAGTTTGTGG
***PR1* R**: TTGGCACATCCGAGTCTCACTG

2.2.16.3 cDNA Amplification and Labelling

First strand cDNA synthesis, second strand cDNA synthesis and SPIA (Single Primer Isothermal Amplification) was performed using the Ovation® Pico WTA System (NuGEN). A total of 50 ng RNA was used as starting material and manufacturers instructions were followed except half quantities were used for each reaction. Amplified cDNA was purified using a Qiagen Qiaquick PCR purification kit. Manufacturers instructions were followed except cDNA was washed with 80 % ethanol in place of the provided wash buffer. Purified cDNA was labelled with Cy3 using the One-Color DNA labelling Kit (Roche), following manufacturer's instructions except half volume reactions were used.

2.2.16.4 Hybridisation

Three and a half μg of labelled cDNA was hybridised to the 12x135k Nimblegen microarray slides (Roche) according to manufacturer's instructions and hybridised overnight at 42 °C.

2.2.16.5 Scanning and Data Analysis

Hybridised slides were washed and dried according to the manufacturer's protocol. Slides were scanned immediately after dying using a MS 200 Microarray Scanner (Roche).

DEVA software (Roche) was used for mapping the array template to the scanned images and generating intensity values per probe. ANAIS (Analysis of NimbleGen Arrays Interface Suite) (Simon and Biot, 2010) was used to perform RMA background correction and inter-array normalisation using quantile normalisation. The 'Hypergeometric Motif Test' tool was used as part of the Analysis of Plant Promotor-Linked Elements (APPLES) software suite developed by Laura Baxter et. al., personal communication.

2.2.17 *Arabidopsis thaliana* mesophyll protoplasts

A. thaliana mesophyll protoplasts were made as described in (Yoo et al., 2007). Briefly, leaf 5, 6 and 7 was removed from 3 week old *A. thaliana* plants and thinly sliced. Sliced leaves were placed in enzyme solution (Mannitol and Macerozyme) and vacuum infiltrated before leaving in the dark for 4 hours. Protoplasts were counted using a haemocytometer and re-suspended at a concentration of $2x10^5$ / ml.

A total of 6 μg DNA was mixed into 2 ml microfuge tube tube, including effector and 35S::Luciferase which is used as control to measure transformation efficiency. 100 μl of protoplasts and an equal volume of fresh PEG solution (40% PEG4000, 0.2 M mannitol and 100 mM $CaCl_2$) was added before incubation for 10 minutes. To stop transfection, 500 μl of cold W5 solution (2 mM MES, 154 mM NaCl, 125 mM, $CaCl_2$ and 5 mM KCl, pH5.7) was added. Protoplasts were centrifuged at 100 G for 2 minutes and resuspended in W1 solution (4 mM MES, 0.5 M mannitol, 20 mM KCl, pH 5.7) before overnight incubation in the dark.

One hundred μM SA dissolved in MeOH or MeOH control was added to protoplast suspensions and incubated for 6 hours in the dark. Protoplasts were then collected into a 1.5 ml microfuge tube and centrifuged at 17000 G for 1 minute, the supernatant was removed. Protoplasts were lysed by the addition of 100 μl of lysis buffer (Luciferase Assay System; Promega catalogue number E1500) and vortexing before centrifugation to pellet cell debris.

Luciferase Assay

20 μl of lysate was transferred into a white opaque flat bottom 96 well plate. To each sample, 20 μl Luciferase assay buffer (Luciferase Assay System; Promega catalogue number E1500) was added. Luminescence was measured using a TECAN GENios Microplate Reader.

PR1::GUS Assay

10 μl of lysate was incubated with 100 μl of MUG substrate mix (10 mM Tris-HCl (pH 8), 1 mM 4-methylumbelliferyl β-D-glucuronide (MUG) and 2 mM $MgCl_2$) for 60 minutes at 37 °C. The reaction was halted by the addition of 900 μl 0.2 M Na_2CO_3. Fluorescence was measured in a clear, flat bottom 96 well plate using a plate reader. Measurements were made in triplicate using a TECAN GENios Microplate Reader with a 360 nm excitation filter and 465 nm emission filter. Relative GUS measurements were then calculated based on luciferase luminescence.

Chapter 3

The effect of HaRxL21 on pathogenicity of a range of phytopathogens

3.1 Introduction

The immune responses of plants against phytopathogens can be broadly categorised into PAMP triggered immunity (PTI) and the recognition of pathogen effector proteins known as effector triggered immunity (ETI). There are many examples of pathogen effector proteins which compromise the ability of the plant to defend itself against disease, for example the *Magnaporthe oryzae* effector Slp1 which compromises PTI in rice (Mentlak et al., 2012). Initial screens have shown that the presence of HaRxL21 causes enhanced susceptibility to phytopathogens (Fabro et al., 2011). The work here aims to validate these results and further characterise the effect of HaRxL21 on the host plant.

During ETI, effector proteins are recognised by 'R-genes' in the host plant, either in a direct 'gene-for-gene' interaction, or through the monitoring of effector targets known as the guard hypothesis (Flor, 1971; McDowell and Woffenden, 2003). There are two R-gene mediated signalling pathways in *A. thaliana*, brought about by contrasting R gene types; the coiled coil (or CC) type and those which are similar to *Drosophila* Toll and mammalian interleukin 1 transmembrane receptors, known as 'TIR' type (Aarts et al., 1998).

It has been shown that knocking out TPL-RELATED 1 (TPR1) compromises immunity brought about by several TIR-NB-LRR type R genes including SNC1 (Zhu et al., 2010). HaRxL21 has been found by yeast-two-hybrid to interact with TPL (Mukhtar et al., 2011); which has 95 % similarity at the amino acid level (Zhu et al., 2010) to TPR1. It is there-

fore logical to investigate whether the presence of HaRxL21 results in disruption of ETI, particularly when brought about by TIR-NB-LRR type R genes.

3.1.1 Experimental Approaches

In order to determine whether HaRxL21 is important for pathogenicity of *Hpa* it would be ideal to produce a *Hpa* mutant with HaRxL21 knocked out and observe whether virulence is compromised. However due to the nature of *Hpa* as an obligate biotroph it cannot be transformed, therefore in order to determine whether HaRxL21 has a effect on host response to a range of pathogens it was expressed and studied *in planta*. Prior to the start of this prom ext,*A. thaliana* lines were generated that have been transformed with pB2GW7; a Gateway® destination vector for expression of the gene of interest under the 35S promoter from Cauliflower mosaic virus (Karimi et al., 2002). This vector contains a Basta (a glufosinate herbicide) resistance gene for transgenic plant selection which has enabled the selection of homozygous lines which have been selected over four generations and used for this study. These lines have been designated HaRxL21a/b/c and are derived from independent transformation events. Details of plant lines used can be found in section 2.1.7.

Transient expression of HaRxL21 in *Nicotiana benthamiana* (*Nb*) is another method by which pathogenicity effects in the presence of the effector can be investigated. This method was used by Bos et al. (2010) to study the *Phytophthora infestans* (*Pi*) effector AVR3a. Here, this method is used to establish whether HaRxL21 is able to alter susceptibility to *Pi*, of which *A. thaliana* is a non-host.

3.1.2 Aims

The aims of this chapter are to establish whether the presence of the effector alters:

1. *Arabidopsis thaliana* growth phenotype.
2. Susceptibility to *Hyaloperonospora arabidopsidis* infection.
3. Susceptibility to *Phytophthora infestans* infection.
4. Susceptibility to *Botrytis cinerea* infection.
5. Susceptibility to *Pseudomonas syringae* infection.
6. Effector triggered immunity as measured by compromised resistance phenotypes or ion leakage due to the hypersensitive response.

3.2 Phenotype of plant lines

Arabidopsis thaliana lines constitutively expressing HaRxL21 (HaRxL21a, b and c) (detailed in section 2.1.7) were grown in short day conditions for seven weeks. As a control, a line was grown which was transformed with the same vector as the effector but containing the GUS protein (designated Col-0 GUS), therefore allowing any effects of constitutive protein expression using this vector to be examined and distinguished from effects of HaRxL21. The phenotype of all three HaRxL21 expressing lines was observed to be smaller with more rounded leaves than wild type plants (Figure 3.1). This effect is not an artefact of transformation because it is observed (to different extents) in all three independently transformed plant lines and not when the GUS protein is transformed into Col-0 plants. HaRxL21a displays the smallest phenotype, with HaRxL21c displaying the phenotype most similar to the wild type.

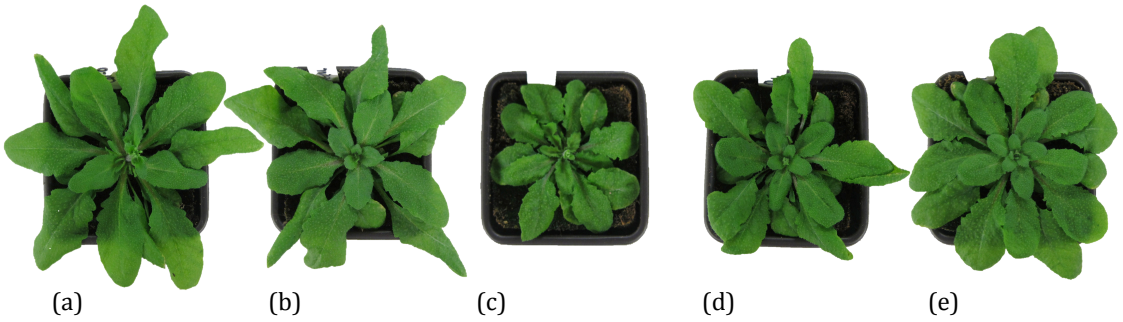


Figure 3.1: *Arabidopsis thaliana* plants expressing 35S::HaRxL21 show a small and round leafed phenotype compared to wild type.
Phenotypic variation between seven week old *Arabidopsis thaliana* plants; (a) Col-0 wild type and (b) Col-0 GUS control compared to lines constitutively expressing HaRxL21; (c) HaRxL21a, (d) HaRxL21b and (e) HaRxL21c, which appear to show a small phenotype with more rounded leaves.

Expression of *HaRxL21* was determined using quantitative reverse-transcription PCR (qRT-PCR) by Jens Steinbrenner. *HaRxL21* expression was normalised to the housekeeping genes *UBQ5* and *AtAct2*. *HaRxL21* expression was detected in all three plant lines, with no significant difference in expression between the lines (Figure 3.2). High *HaRxL21* expression was not found to correlate with severely stunted growth (Figures 3.2 and 3.1).

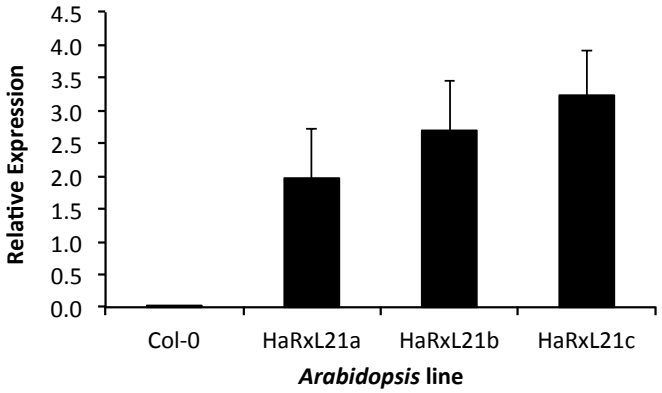


Figure 3.2: Relative expression of *HaRxL21* in 35S::HaRxL21 lines; HaRxL21a-c.
Relative expression of *HaRxL21* was determined by quantitative RT-PCR. Expression of *HaRxL21* in *Arabidopsis thaliana* plants expressing 35S::HaRxL21 (HaRxL21a-c). Expression levels were normalised to *AtAct2* and *UBQ5*. Error bars show standard error between 3 technical replicates. qRT-PCR was performed by Jens Steinbrenner.

This phenotype was then investigated in the context of the interacting proteins identified by Mukhtar et al. (2011); TCP14, TOPLESS (TPL), SWAP and OBE1. This was achieved by literature review and phenotypic observation of available plants. It has been shown that TCP14 and TCP15 play a role in leaf shape (Kieffer et al., 2011), and can be observed that plants with a t-DNA insertion to knock out *TCP14* show a phenotype similar to that observed in HaRxL21 expressing plants (figure 3.3). However, one feature of TCP14 t-DNA insertion plants is that the edges of their leaves curl downwards, not observed in HaRxL21 plants.

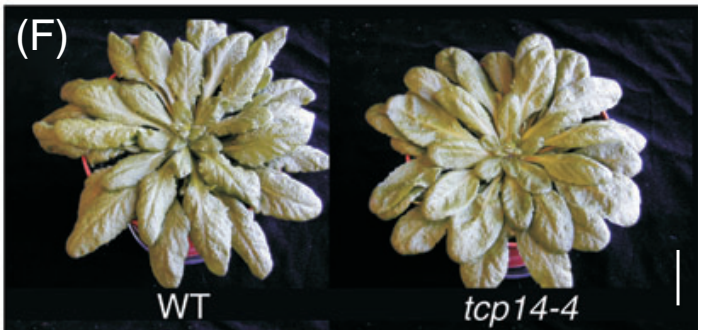


Figure 3.3: An *Arabidopsis thaliana TCP14* T-DNA insertion line show a small and round leafed phenotype compared to wild type (Col-0).
The phenotype of *A. thaliana* wild type (Col-0) compared to a *TCP14* T-DNA insertion line after growth under short day conditions, scale bar represents 2 cm. Adapted from Kieffer et al. (2011), reproduced with permission from Wiley OnlineOpen Library.

The phenotype of *A. thaliana* plants with altered expression of TPL was also investigated. *A. thaliana* Col-0 plants with *TPL*, *TPR1* and *TPR4* knocked out (*tpr1-tpl-tpr4*) were pro-

vided by Zhu et al. (2010). Compared to wild type Col-0 plants (figure 3.1a), these plants showed thinner and more elongated leaves (figure 3.4a). *A. thaliana* plants of the ecotype Landsberg expressing TPL fused to 6xHA tag (provided by Szemenyei et al. (2008)) were grown under the same conditions and showed a leaf phenotype with rounded leaves (figure 3.4b). It has also been shown that over-expression of HA::TPR1 (which shares 95 % homology to TPL at the amino acid level) causes a dwarf phenotype, the severity of which correlates with expression level. These data suggest that leaf morphology is altered by changes in expression of TPL and its related proteins.

(a) **(b)**

Figure 3.4: Changes in expression of the *Arabidopsis thaliana* TOPLESS protein alters leaf phenotype.
Phenotypic variation between six week old *Arabidopsis thaliana* plants grown under short day conditions; (a) *tpr1-tpl-tpr4* and (b) HA::TPL.

3.3 *Hyaloperonospora arabidopsidis* pathogenicity

The effect of HaRxL21 *in planta* was assessed by determining whether *A. thaliana* Col-0 plants constitutively expressing HaRxL21 have altered susceptibility to *Hyaloperonospora arabidopsidis* (*Hpa*). HaRxL21 was originally cloned from the *Hpa* isolate Emoy2, however this isolate cannot be used for pathogenicity screens because *A. thaliana* ecotype Col-0 is resistant to Emoy2 due to the recognition of ATR4 (*Arabidopsis thaliana* recognised 4) by RPP4 (recognition of *Peronospora parasitica* 4). Isolates of *Hpa* which show a compatible growth phenotype on the *A. thaliana* accession Col-0 were used in order to see whether basal defense responses were compromised (as opposed to a compromised resistance mechanism).

3.3.1 Noks1

The *Hpa* isolate Noks1 displays a virulent growth phenotype on plants of the *A. thaliana* Col-0 ecotype (Holub and Beynon, 1997). Noks1 spores were sprayed on two week old *A. thaliana* seedlings expressing 35S::HaRxL21 (HaRxL21a, HaRxL21b and HaRxL21c) as well as Col-0 and Col-0 expressing 35S::GUS, which was used to control for any phenotypic effects due to *in planta* protein expression using the pB7WG2 vector. The *Hpa* spraying method is detailed in section 2.2.6.3. Sporangiophores were counted 4 days post infection; the results from these counts show that *Hpa* isolate Noks1 sporangiophore counts four days post infection are significantly higher on *A. thaliana* lines HaRxL21b and HaRxL21c compared to Col-0 GUS, (P=0.0000057 and 0.00074 respectively using a two tailed, type 2 t-test) (figure 3.5a). Plant from the third independently transformed line (HaRxL21a) show no significant difference (P=0.89).

3.3.2 Maks9

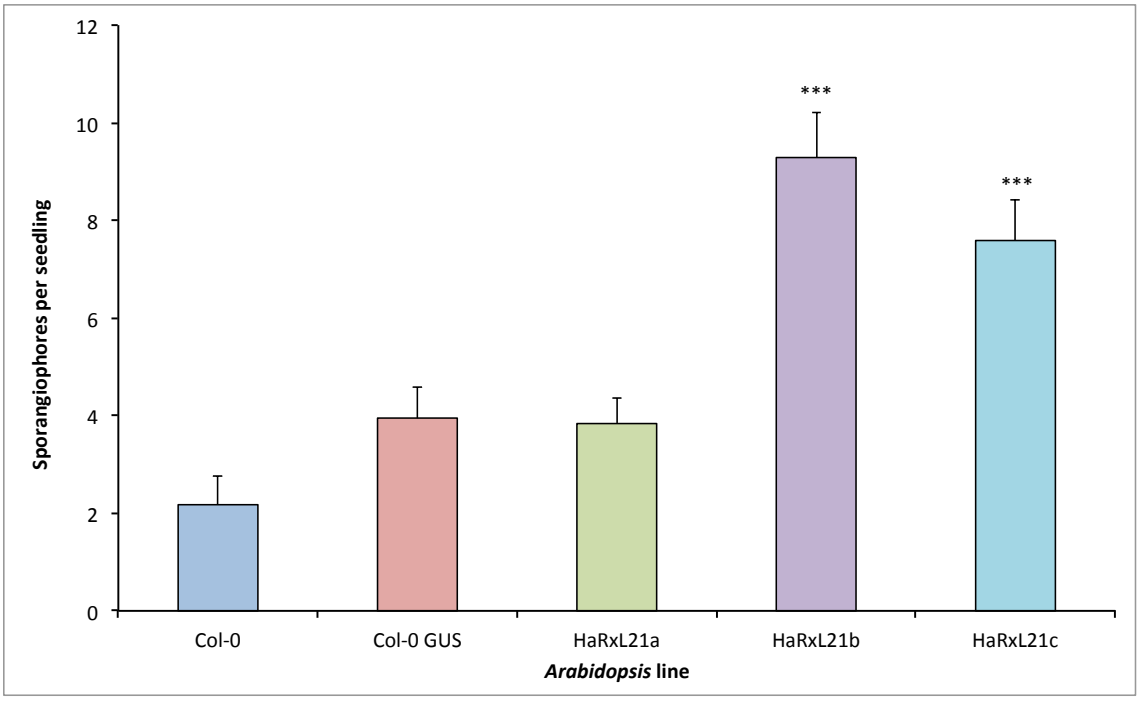
In order to determine whether the susceptibility advantage observed on *A. thaliana* plants expressing 35S::HaRxL21 translated to other compatible isolates of *Hpa*, spores of *Hpa* isolate Maks9 were sprayed onto two week old *A. thaliana* seedlings; HaRxL21a, HaRxL21b and HaRxL21c. Controls used were Col-0 and 35S::GUS in a Col-0 background, in addition to F1/3. The line F1/3 is *A. thaliana* ecotype Col-0 transformed with 35S::HaRxL14, which has consistently shown enhanced susceptibility and is therefore used as a positive control (Jens Steinbrenner, personal communication). The results show that HaRxL21b and HaRxL21c showed enhanced susceptibility to *Hpa* isolate Maks9 (P=0.020 and P=0.0037

respectively using a two-tailed, type 2 t-test), whereas HaRxL21a did not (Figure 3.5b). These results show concurrence with the results obtained during Noks1 infection since HaRxL21a again shows no difference in susceptibility phenotype compared to wild-type plants.

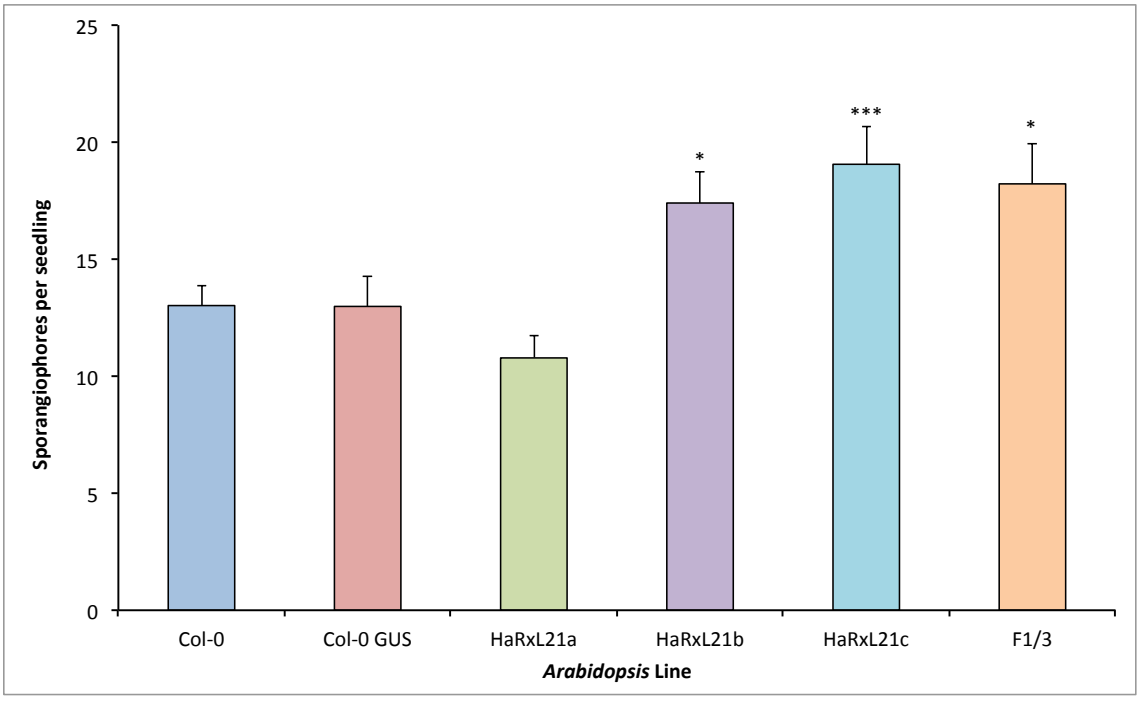
3.3.3 Emco5

Hpa isolate Emco5 is also virulent on the *A. thaliana* ecotype Col-0 (Holub and Beynon, 1997). Therefore in order to determine whether *A. thaliana* plants expressing HaRxL21 were more susceptible to Emco5, spores were sprayed onto two week old *A. thaliana* seedlings; HaRxL21a, HaRxL21b and HaRxL21c, in addition to controls Col-0, 35S::GUS in a Col-0 background and F1/3 positive control for enhanced susceptibility. No change in susceptibility was observed in the presence of HaRxL21 (figure 3.6). There was also no enhanced susceptibility observed on the F1/3 line and low growth overall (mean sporangiophore counts per seedling were below 1.5) which indicated a resistance phenotype.

To confirm this, sporangiophores were counted on seedlings of the *A. thaliana* accession Wassilewskija, an enhanced disease susceptibility mutant (ws-eds) (Parker et al., 1996). This plant line is routinely included in *Hpa* screens to ensure correct disease development has occurred. These seedlings showed significantly higher growth than the Col-0 seedlings (wild type and transgenic), suggesting that low sporangiophore counts were not due to experimental conditions which prevented development of disease.



(a)



(b)

Figure 3.5: Expression of HaRxL21 *in planta* causes enhanced susceptibility to *Hpa* isolates Noks1 and Maks9.

Hyaloperonospora Arabidopsidis spores of isolate (a) Noks1 or (b) Maks9 were sprayed on two week old *Arabidopsis thaliana* seedlings expressing 35S::HaRxL21 (HaRxL21a, HaRxL21b and HaRxL21c) and controls (wild type Col-0 and 35S-GUS in a Col-0 background), in addition to F1/3 as a positive control for enhanced susceptibility. Sporangiophores were counted 4 days post infection using a dissecting microscope. Error bars show standard error and significant differences to Col-0 GUS using a T-test are shown (*p<0.05; **p<0.01; ***p<0.001, n=45).

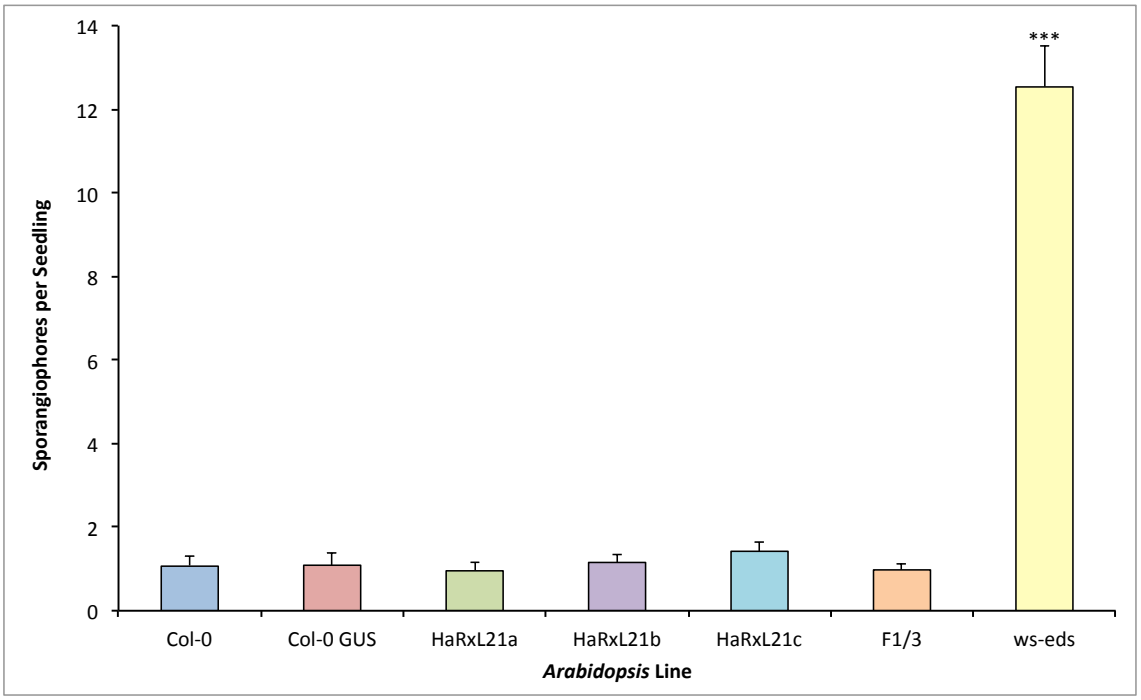


Figure 3.6: Expression of HaRxL21 *in planta* does not enhance susceptibility to *Hpa* isolate Emco5.

Hyaloperonospora arabidopsidis spores of isolate Emco5 were sprayed on two week old *A. thaliana* seedlings expressing 35S::HaRxL21 (HaRxL21a, HaRxL21b and HaRxL21c) and controls (wild type Col-0 and 35S-GUS in a Col-0 background), in addition to F1/3 as a positive control for enhanced susceptibility caused by an effector protein. ws-eds was used as a control to ensure that conditions were favourable for disease development. Sporangiophore counts four days post-infection are shown, with error bars showing the standard error. No significant differences were observed using a T-test (n=45).

3.4 *Phytophthora infestans* pathogenicity

Like *Hpa*, the genome of the oomycete pathogen *Phytophthora infestans* (*Pi*) also contains effector proteins which can be characterised by the presence of the 'RxLR' motif. These pathogens share similar infection strategies although unlike the obligate biotroph *Hpa*, the lifecycle of *Pi* includes a necrotrophic phase, and it is therefore known as a hemibiotroph (Thines, 2009). In order to determine whether the presence of HaRxL21 aids the infection process of this related oomycete pathogen, *Pi* virulence in the presence of this effector was tested.

A. thaliana is a non-host of *Pi*, therefore using 35S::HaRxL21 lines to test for a susceptibility advantage was not possible. Instead, *Agrobacterium tumefaciens* mediated transient transformation of *Nicoiana benthamiana* (*Nb*) was used for *in planta* expression of HaRxL21. The pGRAB vector for un-tagged expression of HaRxL21 (as used for *in planta* expression of AVR3a in Bos et al. (2010)) was used for *A. tumefaciens* mediated transient transformation of *Nb* plants. The P19 protein from tomato bushy stunt virus is commonly used for preventing post-transcriptional gene silencing (Voinnet et al., 2003) during transient expression in *Nb* by *A. tumefaciens*. Here, *A. tumefaciens* expressing the P19 protein were co-infiltrated with *A. tumefaciens* expressing the effector or the control. This is necessary to prevent silencing of the constructs before the *Pi* infection has run its course (7 days).

Nb leaves expressing pGRAB::HaRxL21 and empty pGRAB were infected with *Pi* (expressing tdTomato red fluorescent protein) spores by drop inoculation. Leaves were detached and tdTomato RFP expression was imaged 7 days post infection, in order to visualise the biotrophic leading edge of the pathogen and accurately quantify lesion size. ImageJ software (http://imagej.nih.gov/ij/) was used to quantify lesion area on images. Different treatments on either side of each leaf were compared using a paired T-test and no significant difference in lesion area was observed in the presence of HaRxL21 compared to the empty vector control (Figure 3.7c).

Nb leaves were also transformed with pGRAB::HaRxL21 and pGRAB::HaRxL21ΔLxLxL. The EAR motif (LxLxL) has been previously shown to mediate interaction domain with the protein TOPLESS (Szemenyei et al., 2008; Pauwels et al., 2010; Kagale and Rozwadowski, 2011) which has been found to interact by Y2H with HaRxL21 (Mukhtar et al. (2011); this study (Figure 4.3.1)). To determine whether any observed susceptibility advantage conferred to *Pi* were due to interaction with TPL, pathogenicity effects of the effector lacking this motif were therefore investigated here. Treatments on either side of each leaf were compared using a paired T-test and no significant difference in lesion area

was observed between HaRxL21 and HaRxL21ΔLxLxL (Figure 3.7d).

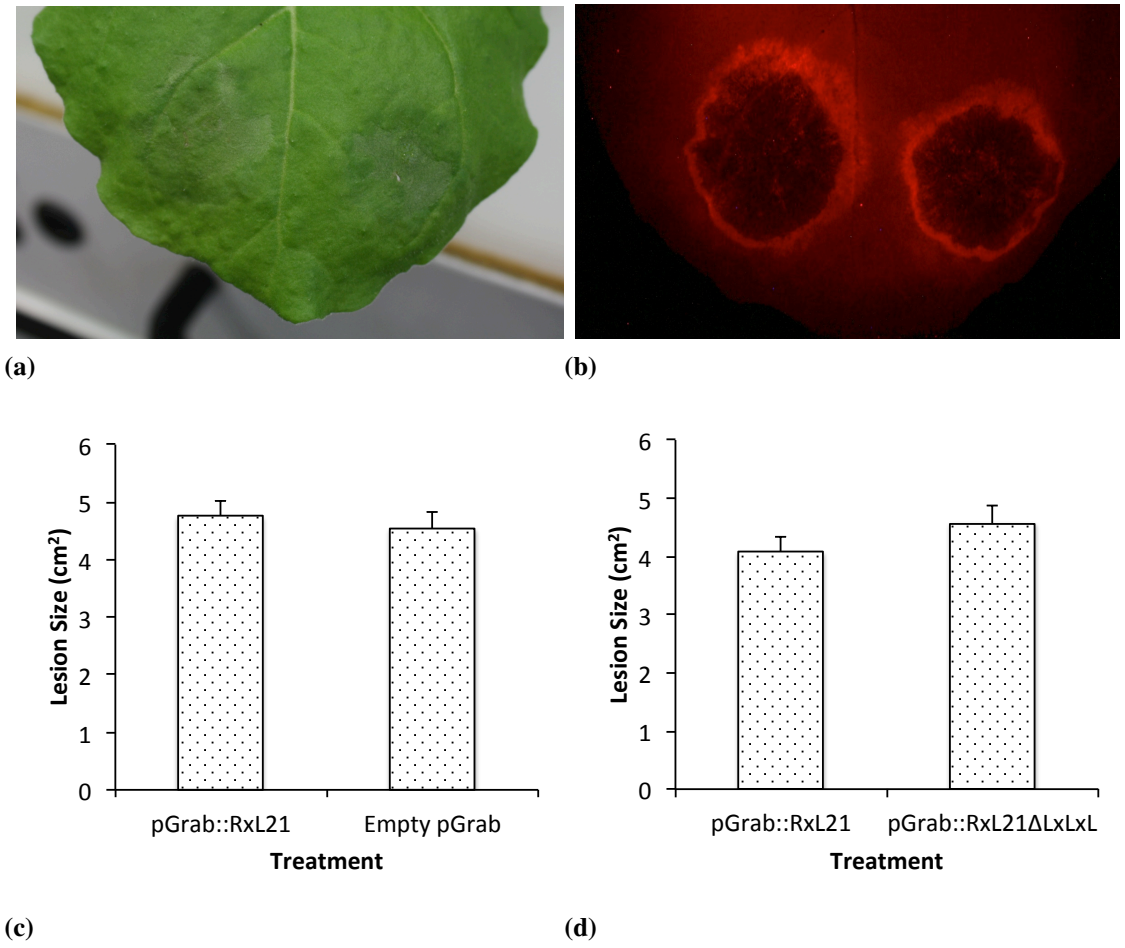


Figure 3.7: Transient *in planta* expression of HaRxL21 does not alter susceptibility of *Nicoiana benthamiana* to *Phytophthora infestans*.
Nicoiana benthamiana plants were infected using a single droplet of a *Phytophthora infestans* tdTomato spore suspension, leaves were subsequently imaged 7 days post infection. The same leaf is shown after imaging by (a) using a standard camera and (b) using RFP excitation. Lesion size as determined by the area in which RFP was expressed was measured using ImageJ software (http://imagej.nih.gov/ij/). Comparisons are shown between pGRAB::HaRxL21 and (c) empty pGRAB or (d) pGRAB::HaRxL21ΔLxLxL. Error bars show standard error, no significant differences were observed between the treatments using a paired T-test (n=72).

3.5 *Botrytis cinerea* pathogenicity

Botrytis cinerea (*B. cinerea*) is a necrotrophic pathogen which uses an infection strategy very different to that of *Hpa* (Glazebrook, 2005). Unlike *Hpa*, it does not require host tissue to be living in order to obtain nutrients. It also means that plants must 'fine tune' their defense responses and respond differently, for example programmed cell death would prevent a biotroph from proliferating, but aid a necrotrophic pathogen.

Six week old *A. thaliana* leaves were drop inoculated with a suspension of *B. cinerea* spores in grape juice, and the infected tissue imaged at 24, 48, 72 and 96 hours post infection. Controls used were Col-0 wild type and Col-0 *npr1* plants, which were used as an additional control because they should show greater resistance than Col-0 to *B. cinerea* due to the removal of SA-mediated JA signalling repression. This is because although the role of the NPR1 protein in the SA response is well documented (see section 1.6.1), NPR1 is also involved in the crosstalk between SA and Jasmonic Acid (JA) signalling; it has been shown that *A. thaliana* plants with *NPR1* knocked out do not show SA-mediated suppression of JA responsive genes (Spoel et al., 2003). During *B. cinerea* infection, PR1 is upregulated compared to mock infection (*Arabidopsis* eFP Browser (Winter et al., 2007)), indicating that some SA signalling is present and likely to antagonise JA responses.

The results from this experiment showed that HaRxL21b was significantly more susceptible to *B. cinerea* infection than Col-0 and Col-0 *npr1* (figure 3.8). HaRxL21a and HaRxL21c appeared to show increased lesion size compared to controls but this was not statistically significant.

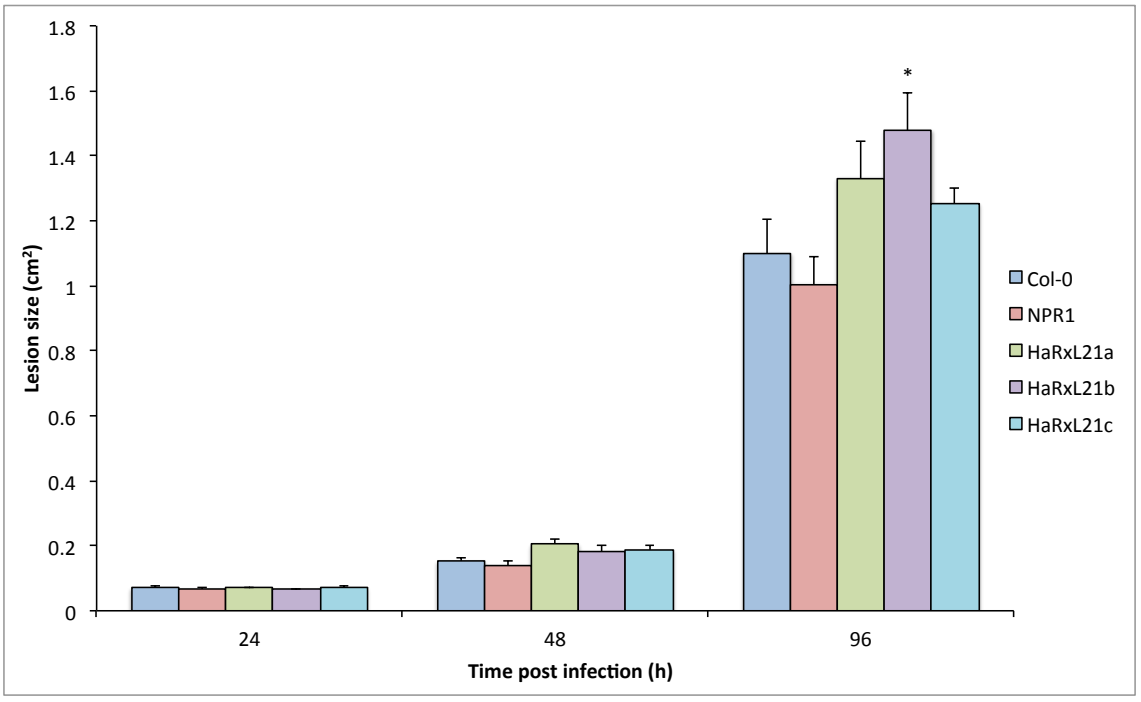
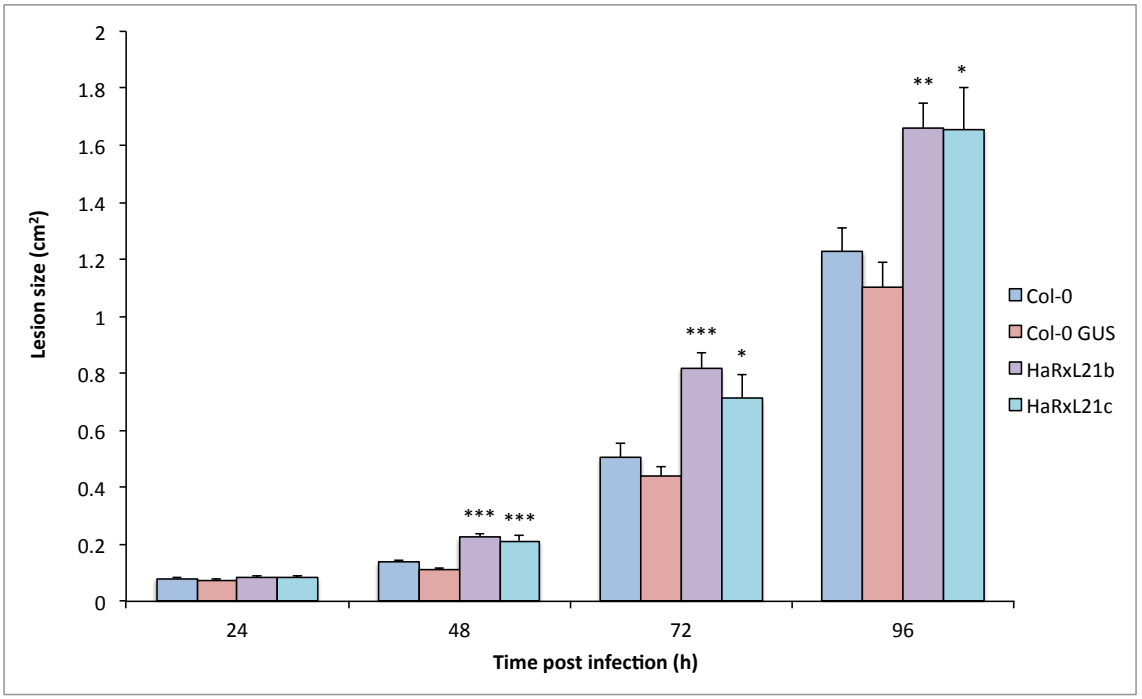


Figure 3.8: Expression of 35S::HaRxL21 *in planta* causes enhanced susceptibility to *Botrytis cinerea*.

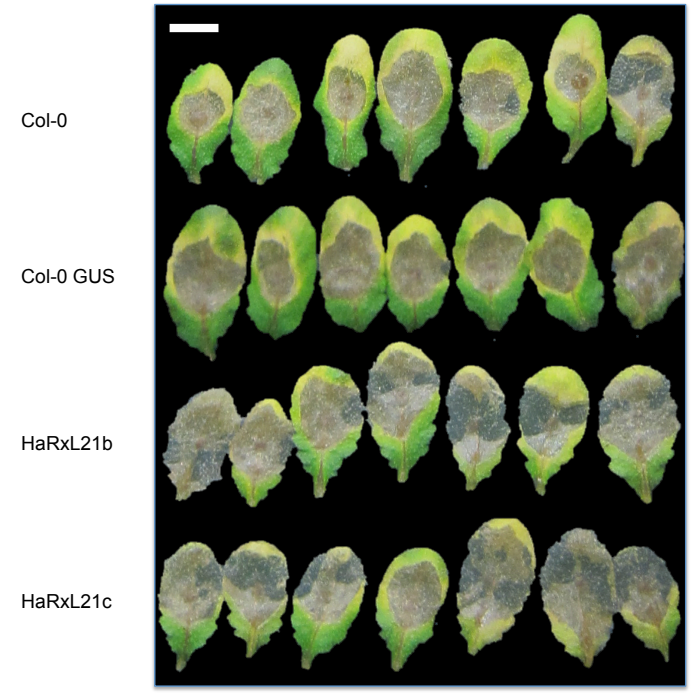
Detached leaves from six week old *A. thaliana* plants expressing 35S::HaRxL21 (HaRxL21a and HaRxL21b) and controls (wild type (Col-0) and Col-0 35S:GUS) were infected with *Botrytis cinerea*, images were taken and lesion area measured at 24, 48 and 96 hours post infection. Significant differences to Col-0 using a T-test are shown (*p<0.05; **p<0.01; ***p<0.001, n=24), error bars display standard error.

The experiment was repeated, with Col-0 GUS plants used as controls to ensure that any difference in susceptibility from Col-0 is not due to the transformation process during the generation of these transgenic plants, or due to constitutive expression of a protein. Two 35S::HaRxL21 *A. thaliana* lines were used; HaRxL21b and HaRxL21c. Using only two lines enabled greater replication within plant growth constraints, these lines were chosen over HaRxL21a as it's small growth phenotype (figure 3.1) may cause *B. cinerea* growth to be hindered when reaching the edge of the leaves.

The results from this experiment showed that both HaRxL21b and HaRxL21c were significantly more susceptible to *B. c* infection than Col-0, when determined by lesion area at 48, 72 and 96 hours post infection (figure 3.9a). This result can also be observed visually (figure 3.9b). HaRxL21b and c also showed significantly larger lesion areas than Col-0 GUS at all time points ($P<0.001$). There was no significant difference between lesion area on Col-0 and Col-0 GUS plants.



(a)



(b)

Figure 3.9: Expression of 35S::HaRxL21 *in planta* causes enhanced susceptibility to *Botrytis cinerea*.

Detached leaves from six week old *A. thaliana* plants expressing 35S::HaRxL21 (HaRxL21a and HaRxL21b) and controls (wild type (Col-0) and Col-0 35S:GUS) were infected with *Botrytis cinerea*, images were taken and lesion size measured at 24, 48 and 96 hours post infection. (a) Mean lesion area and (b) Representative leaf images at 96 hours post infection are shown. Significant differences to Col-0 are shown (*p<0.05; **p<0.01; ***p<0.001), error bars display standard error (n=24). Scale bar in (b) is equal to 1 cm.

3.5.1 *Botrytis cinerea* pathogenicity in *Nicotiana benthamiana*

Since *B. cinerea* is able to infect *Nb*, one aim here was to establish a system which would allow screening for *B. cinerea* pathogenicity effects of effector proteins transiently expressed in *Nb*. This would result in a system where truncated or mutated forms of effectors could be rapidly screened for pathogenicity effects without the need to generate stably transformed *A. thaliana* lines.

First, it was necessary to establish whether the presence of *Agrobacterium tumefaciens* would hinder *B. cinerea* growth due to the induction of plant defenses. *Nb* plants were infiltrated with *A. tumefaciens* expressing the P19 silencing suppressor and this was compared to infiltration with infiltration buffer (as described in section 2.2.5.3). The P19 silencing suppressor is particularly important here as without this silencing suppressor, effector expression in *Nb* would be limited to 2-3 days; before the *B. cinerea* infection time course is complete. P19 was present in all subsequent infiltrations and was therefore used in these initial experiments. Comparisons were made by applying each treatment to either side of the same leaf, then using a paired T-test to determine whether the lesion sizes were significantly different to each other. Using this method, inter-leaf variability is taken into account. It was shown that the presence of *A. tumefaciens* expressing P19 reduces *Nb* susceptibility to *B. cinerea* when compared to infiltration buffer at 48, 72 and 96 hours post infection (figure 3.10).

It was then investigated whether the transient expression of HaRxL21 could increase the susceptibility of *Nb* leaves to *B. cinerea* infection. This was done by infiltrating leaves with *A. tumefaciens* expressing P19 and either HA::HaRxL21 or HA::GFP, then infecting with *B. c* spores at 500 000 spores / ml by drop inoculation. It was again necessary to make binary comparisons for this study to account for variation in expression levels between *Nb* leaves; in this case it was possible to make direct comparisons by comparing the two treatments on either side of the same leaf. It was found that there was no significant difference in susceptibility due to the presence of HaRxL21 (Figure 3.11a). The data suggests that larger lesion sizes could be observed in the presence of HaRxL21 48 h post infection, although this was not statistically significant using a two-tailed paired t-test (P=0.094). Seventy two hours post infection, lesions appeared larger on areas expressing HA::GFP, it was observed that this appeared to be due to arresting of the lesions on transient HA::HaRxL21 expression.

The experiment was repeated with a higher number of infected leaves to account for variability, and a lower spore concentration (200 000 spores / ml) to ensure that any phenotypic effects of HaRxL21 were not hidden by the large inoculum of *B. cinerea*. No sig-

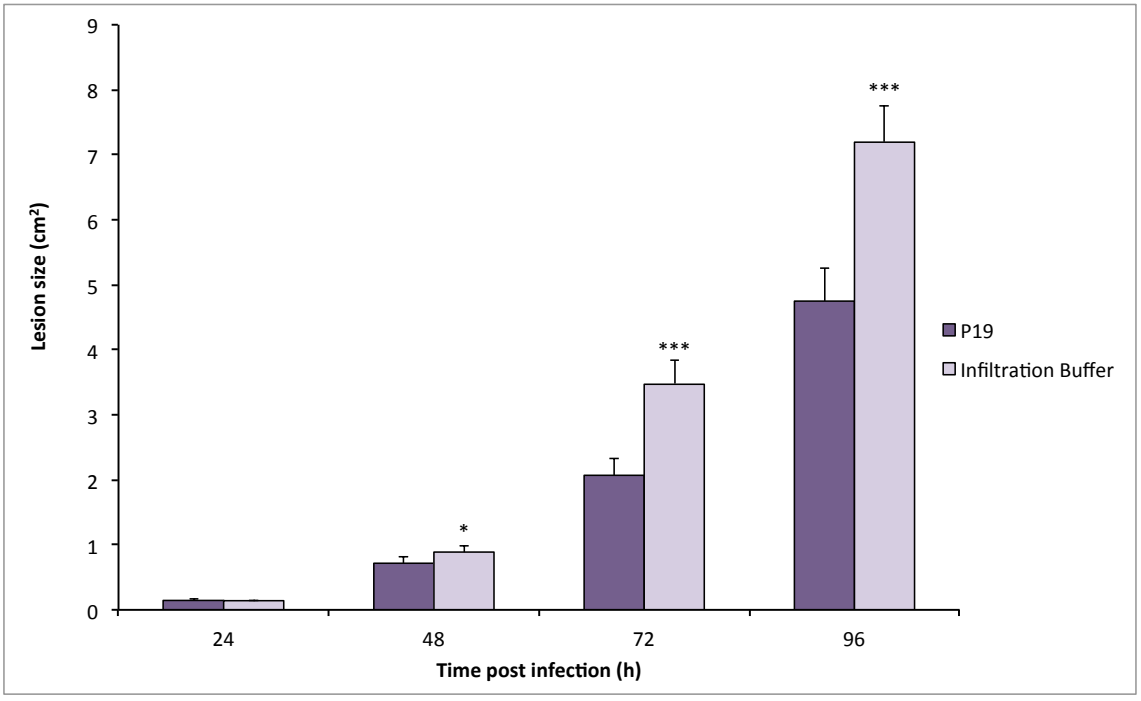
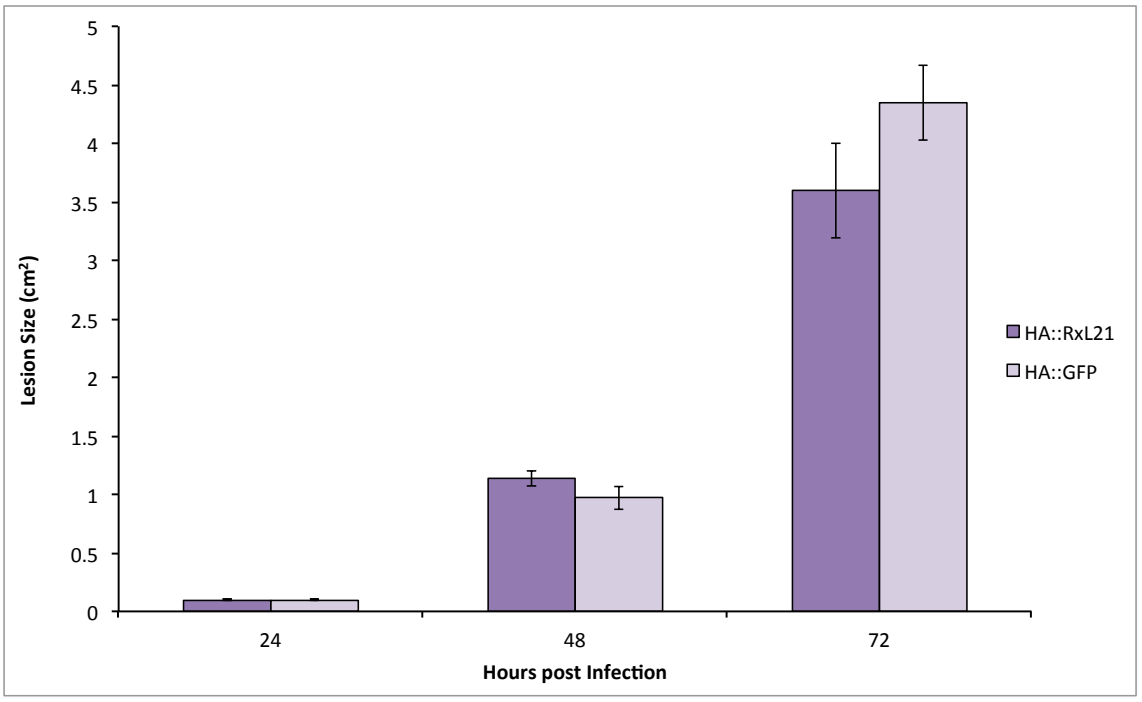


Figure 3.10: Infiltration with *Agrobacterium tumefaciens* causes reduced susceptibility of *Nicotiana benthamiana* to *Botrytis cinerea*.

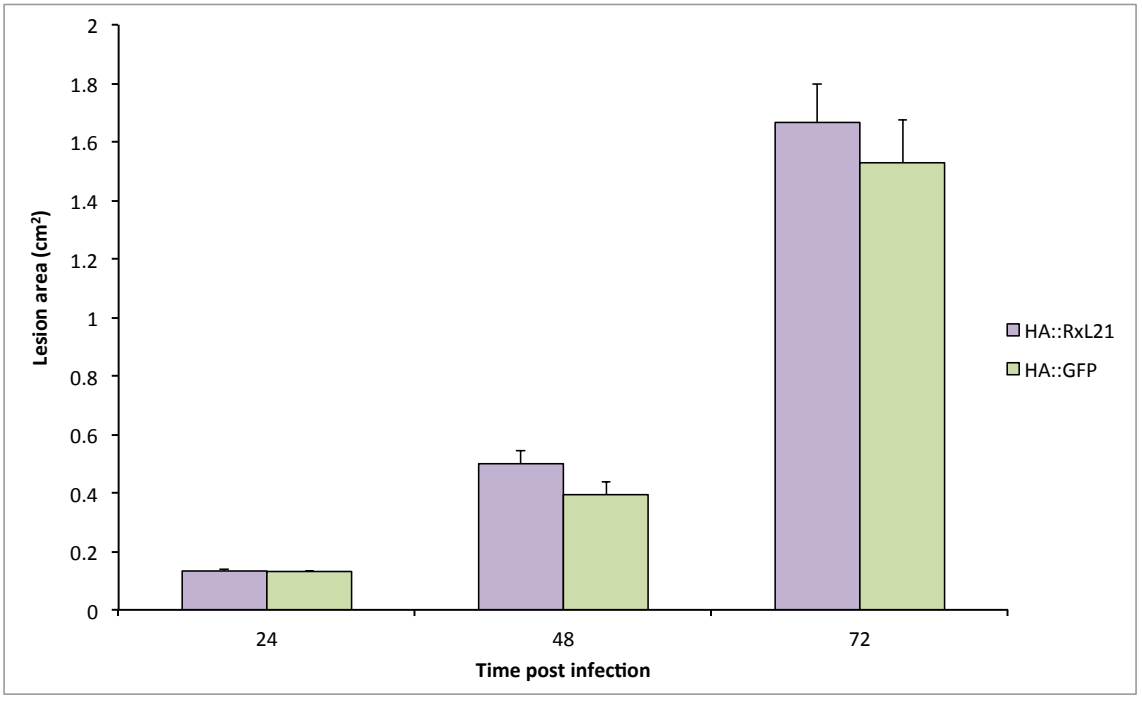
Nicotiana benthamiana was infiltrated with *A. tumefaciens* expressing P19 silencing suppressor or with infiltration buffer on opposite sides of each leaf. Comparisons were made between lesions area on either side of the same leaf, using paired t-test to determine whether there was a significant difference in lesion size. (*p<0.05; **p<0.01; ***p<0.001). Error bars display standard error, n=13.

nificant difference was observed between lesions on leaf areas expressing HA::HaRxL21 compared to HA::GFP 3.11b.

It is possible that the presence of a HA tag might alter the folding of an effector protein or block any biological function in the host cell. To determine whether the vector used for effector delivery had an impact on *B. cinerea* susceptibility, a comparison was made between HaRxL21 delivery via pEarleygate201 (N-terminally HA Tagged) and HaRxL21 delivery via pGRAB; an un-tagged vector as used for *Phytophthora infestans* pathogenicity assays. It was found that at 72 h post infection, lesions on *Nb* leaf areas expressing pGRAB::HaRxL21 were significantly larger than in the presence of HA::HaRxL21 (determined using a paired, two-tailed t-test, P=0.0084) (figure 3.12).



(a)



(b)

Figure 3.11: Transient expression of HaRxL21 *in planta* does not alter *Nicotiana benthamiana* susceptibility to *Botrytis cinerea*.
Nicotiana benthamiana transiently expressing HA::HaRxL21 and HA::GFP, followed by drop inoculation with *Botrytis cinerea* at (a) 500 000 spores / ml (n=17) or (b) 200 000 spores / ml (n=39). Comparisons were made between sizes of lesions on either side of the same leaf using a T-test and no significant difference was observed. Lesion area is shown with error bars showing the standard error. No significant difference was observed between treatments using a paired T-test.

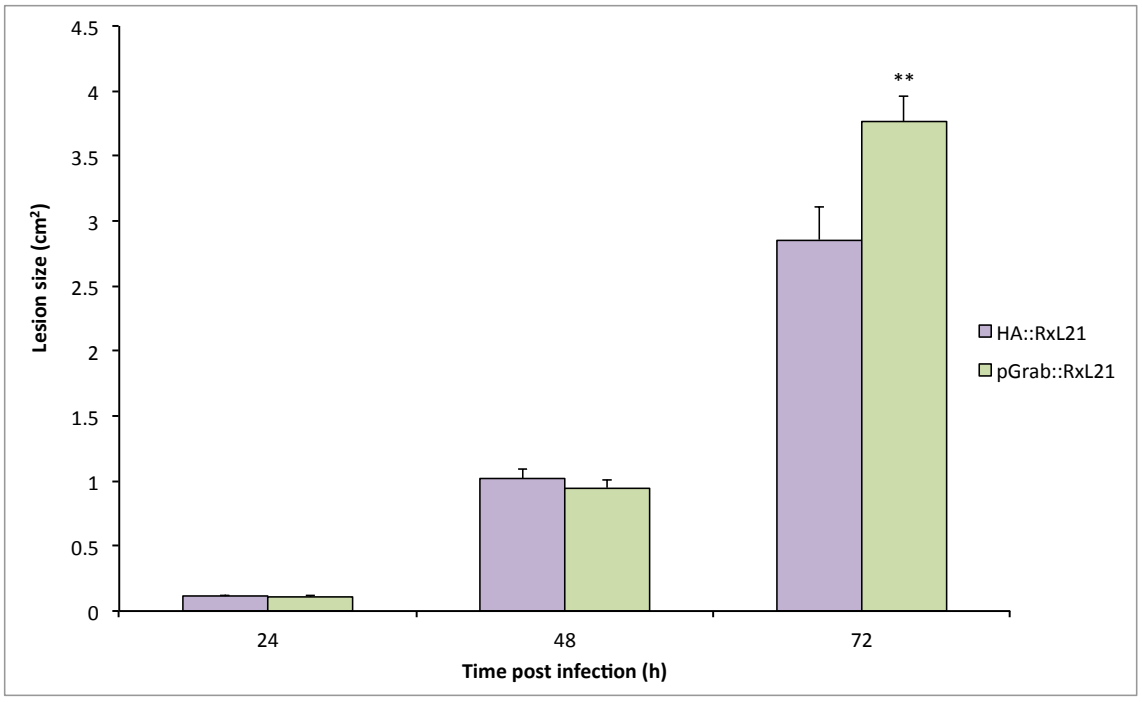


Figure 3.12: Expression vector has an impact on *Nicotiana benthamiana* susceptibility to *Botrytis cinerea*

Nicotiana benthamiana was infiltrated with *A. tumefaciens* expressing HA::HaRxL21 and pGRAB::HaRxL21 on opposite sides of each leaf, followed by drop inoculation with *Botrytis cinerea* spores. Comparisons were made between lesions area on either side of the same leaf, using paired t-test to determine whether there was a significant difference in lesion size. (*p<0.05; **p<0.01, n=18). Error bars show standard error.

3.6 *Pseudomonas syringae* pathogenicity

The effector detector vector (EDV) system (Sohn et al., 2007) can be used to test the individual contributions of effector proteins, delivered through the type III secretion system of *Pseudomonas syringae*. It has previously been shown that delivery of HaRxL21 (by *P. syringae* pv tomato -LUX using the EDV system) promoted bacterial growth in 8 of 12 *Arabidopsis* accessions tested (Fabro et al., 2011).

The aim here was to establish this system for HaRxL21, so that subsequently the screen could be performed on knockout plants for protein targets of HaRxL21, to determine whether individual protein-protein interactions were important for virulence function of this effector. The EDV6 vector containing HaRxL21 was transformed into *P. syringae* by triparental mating (Georgina Fabro, personal communication), since unlike DH5α (chemically competent *E. coli*), *P. syringae* cannot be transformed by heat shocking. Leaves of Col-0 were then infiltrated with *P. syringae* expressing this construct, and colony counts performed to quantify bacterial growth.

Both *Pst lux* alone and *Pst lux* expressing AvrRPS4-AAAA were used as controls. AvrRPS4 is a *P. syringae* effector protein in which the 'KRVY' motif is functional and required for virulence (Sohn et al., 2009). When this motif is mutated to alanine residues, virulence function is removed and it can be used as a control for effector studies, for example in Fabro et al. (2011).

The delivery of HaRxL21 by *Pst lux* was found to slightly enhance bacterial growth compared to AvrRPS4-AAAA control, but not compared to *Pst lux* alone at 72h host infection (figure 3.13).

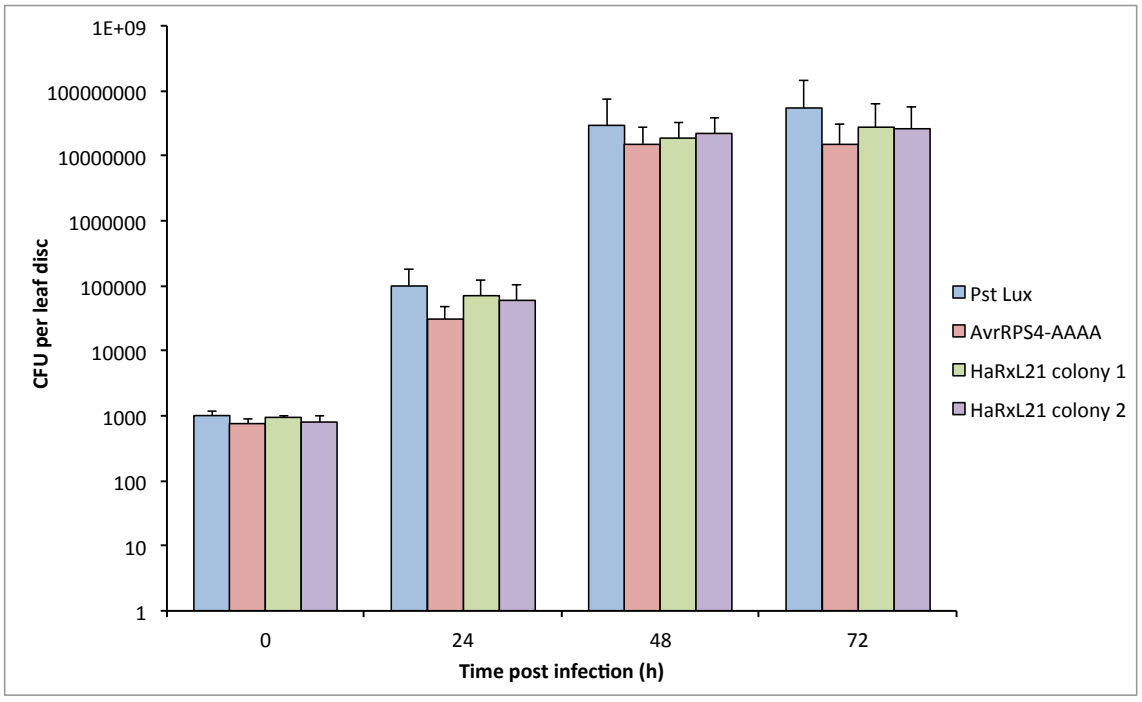


Figure 3.13: Growth of *Pst lux* growth in *A. thaliana* expressing HaRxL21 and compared to *Pst lux* alone and AvrRPS4-AAAA control

Arabidopsis thaliana was infiltrated with *P. syringae* pv tomato lux (1) alone, (2) expressing EDV6 containing avrRPS4-AAAA, (3) and (4) two independently transformed bacterial colonies generated through triparental mating and expressing HaRxL21 in the EDV6 vector. Colony counts taken sampled from 4 pooled leaves are shown using a logarithmic scale, error bars show standard error. No significant difference was observed between the treatments using a T-test (n=6).

3.7 Does HaRxL21 cause disruption of ETI?

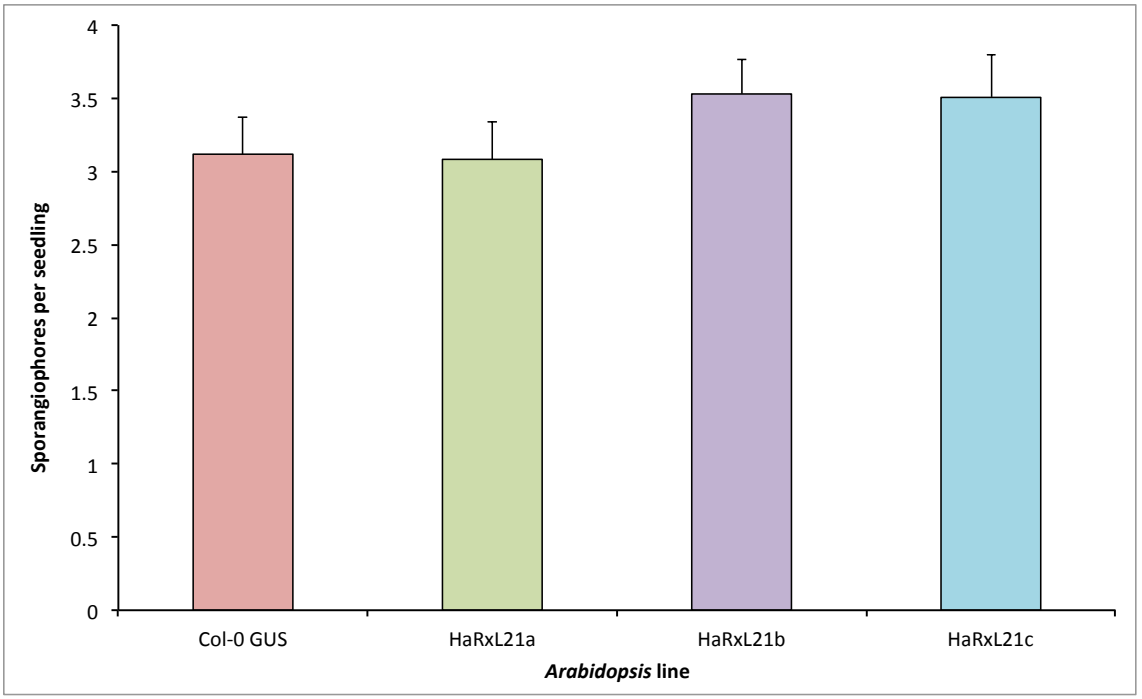
In order to determine whether HaRxL21 is involved in effector triggered immunity (ETI), the following questions were addressed:

1. Does *in planta* expression of HaRxL21 compromise *Hpa* resistance phenotypes?
2. Does *in planta* expression of HaRxL21 prevent ion leakage caused by the hypersensitive response in response to the delivery of *Pseudomonas syringae* avirulence genes?

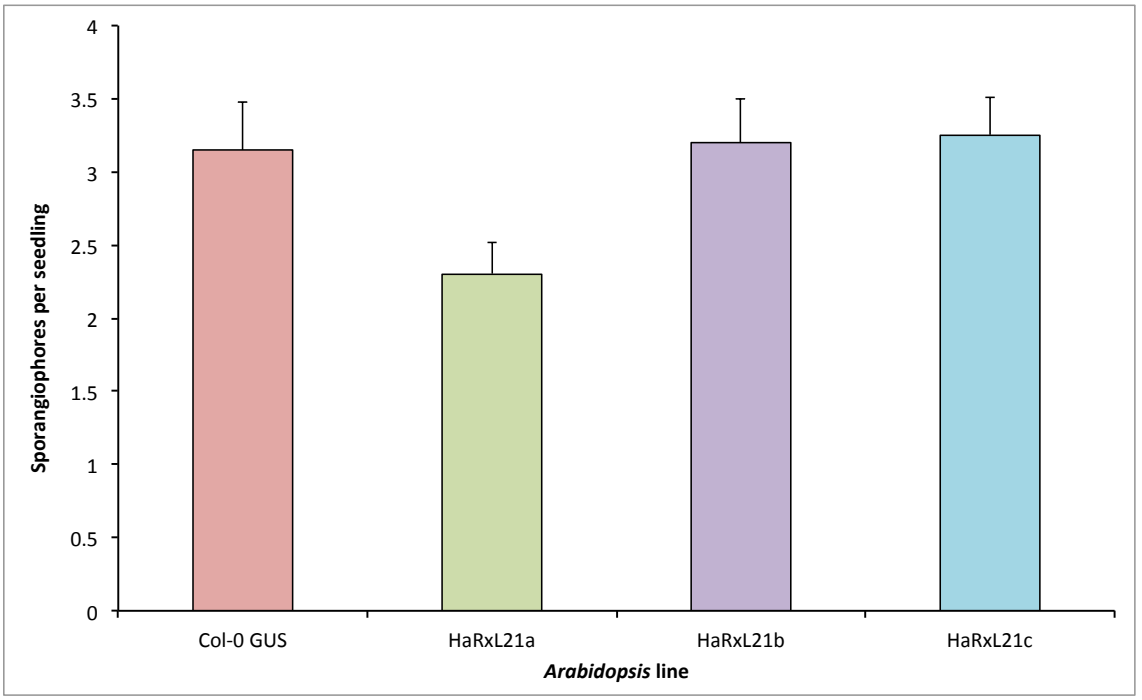
3.7.1 *Hyaloperonospora arabidopsidis* resistance disruption?

One of the reasons why the relationship between *Hpa* and *A. thaliana* is used as a model system for plant pathogen interactions is because there is a naturally occurring variation in resistance phenotypes, as different *A. thaliana* ecotypes vary in their resistance to *Hpa* isolates. The *A. thaliana* ecotype Col-0 is resistant to *Hpa* isolate Emoy2 due to the recognition of ATR4 by RPP4, a TIR-NB-LRR protein (van der Biezen et al., 2002). In order to determine whether HaRxL21 interferes with the mechanism behind this resistance, spores of Emoy2 were sprayed onto seedlings of HaRxL21a, b and c, with Col-0 GUS as a control. To aid sporangiophore counts to quantify susceptibility, the seedlings were 10 days old when infected; younger than the 14 day old seedlings routinely used for screening of virulent isolates. This is because more pathogen growth can observed on younger seedlings (Daniel Tome, personal communication). It was determined that RPP4 mediated resistance was not compromised by the presence of HaRxL21, shown by two independent repeats of the pathogenicity screen (figure 3.14).

The *A. thaliana* ecotype Col-0 is resistant to *Hpa* isolate Hiks1 due to the recognition of ATR7 by RPP7, a CC-NB-LRR protein (Eulgem et al., 2007). To establish whether this resistance was compromised, spores of Hiks1 were sprayed onto 10 day old *A. thaliana* seedlings and sporangiophores counted 5 days post infection. This resistance was not shown to be compromised by two independent repeats of a Hiks1 pathogenicity screen (figure 3.15). The results of the second screen (figure 3.15b) show that HaRxL21b had significantly less sporangiophores per seedling than Col-0 GUS. However the results from these screens were quite variable due to the small numbers of sporangiophores observed on each seedling, and this reduction in susceptibility was only observed in one experiment.



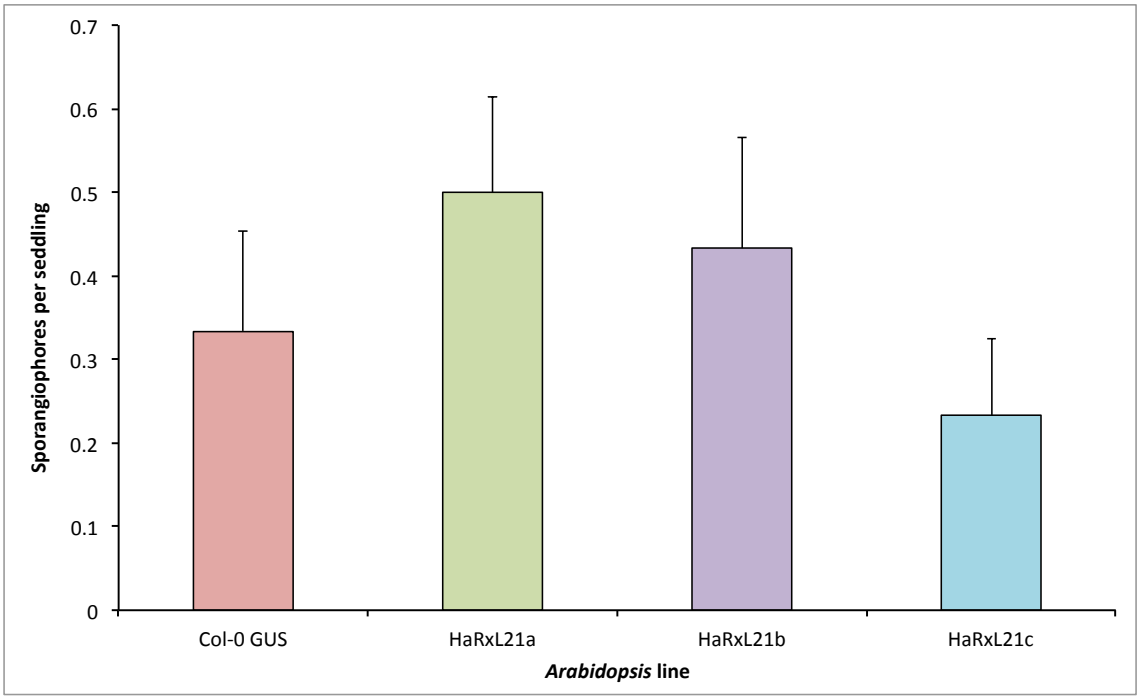
(a)



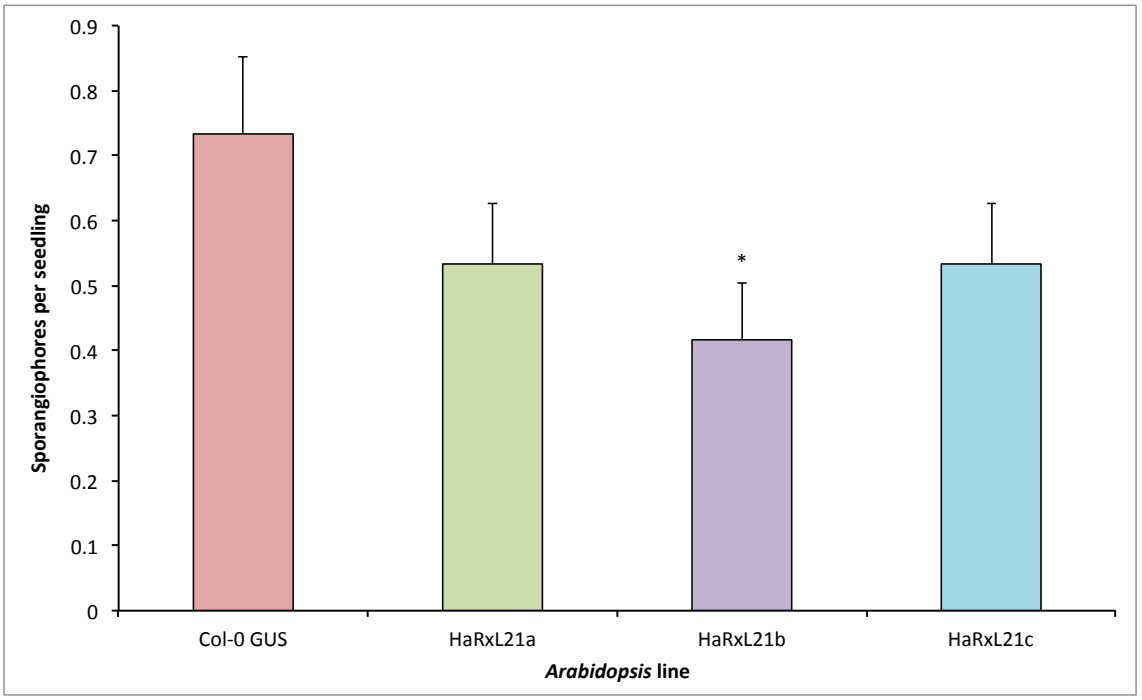
(b)

Figure 3.14: Expression of HaRxL21 *in planta* does not compromise RPP4 mediated resistance to *Hpa* isolate Emoy2.

Pathogenicity of *Hyaloperonospora Arabidopsidis* isolate Emoy2 on three 35S::HaRxL21 lines compared to 35S::GUS, all in a Col-0 background. Emoy2 is incompatible for growth on Col-0 due to the recognition of ATR4 by the resistance protein RPP4. Ten day old *A. thaliana* seedlings were sprayed with *Hpa* spores and resulting sporangiophores were counted 5 days post infection. Results from two independent repeats (a) and (b) are shown. No significant difference was observed using a T-test, n=45.



(a)



(b)

Figure 3.15: Expression of HaRxL21 *in planta* does not compromise RPP7 mediated resistance to *Hpa* isolate Hiks1.

Pathogenicity of *Hyaloperonospora Arabidopsidis* isolate Hiks1 on on three independently transformed 35S::HaRxL21 lines (HaRxL21a-c) compared to 35S-GUS, all in a Col-0 background. Ten day old *A. thaliana* seedlings were sprayed with *Hpa* spores resulting sporangiophores were counted 5 days post infection. Results from two independent repeats (a) and (b) are shown. Error bars display standard error, n=45. Significant differences to Col-0 using a T-test are shown (* = $P<0.05$).

3.7.2 Conductivity Assays

One method by which programmed cell death can be measured is by monitoring conductivity changes due to ion leakage, as observed in Jamir et al. (2004). In Col-0 plants, the the *Pseudomonas syringae* effector proteins AvrRPM1 and AvrRPS4 are recognised by the corresponding 'R' proteins; RPM1 (CC-NB-LRR type) and RPS4 (TIR-NB-LRR type). A pilot experiment was performed to test the experimental procedure and also determine the expected differences in conductivity when Col-0 plants were infiltrated with *Pseudomonas fluorescens* (*PFO*) (a *Pseudomonas* strain engineered with a T3SS for the specific delivery of certain effectors (Thomas et al., 2009)) expressing the *P. syringae* effector proteins AvrRPM1 and AvrRPS4. Conductivity changes were measured over time as described in section 2.2.9.

The pilot experiment showed that measuring conductivity to observe ion leakage was a valid method to observe programmed cell death due to R-protein mediated defense (figure 3.16). It can also be observed that ion leakage due to recognition of avrRPS4 was much higher than that due to AvrRPM1 and that most of the changes in conductivity were in the initial few hours post-infection.

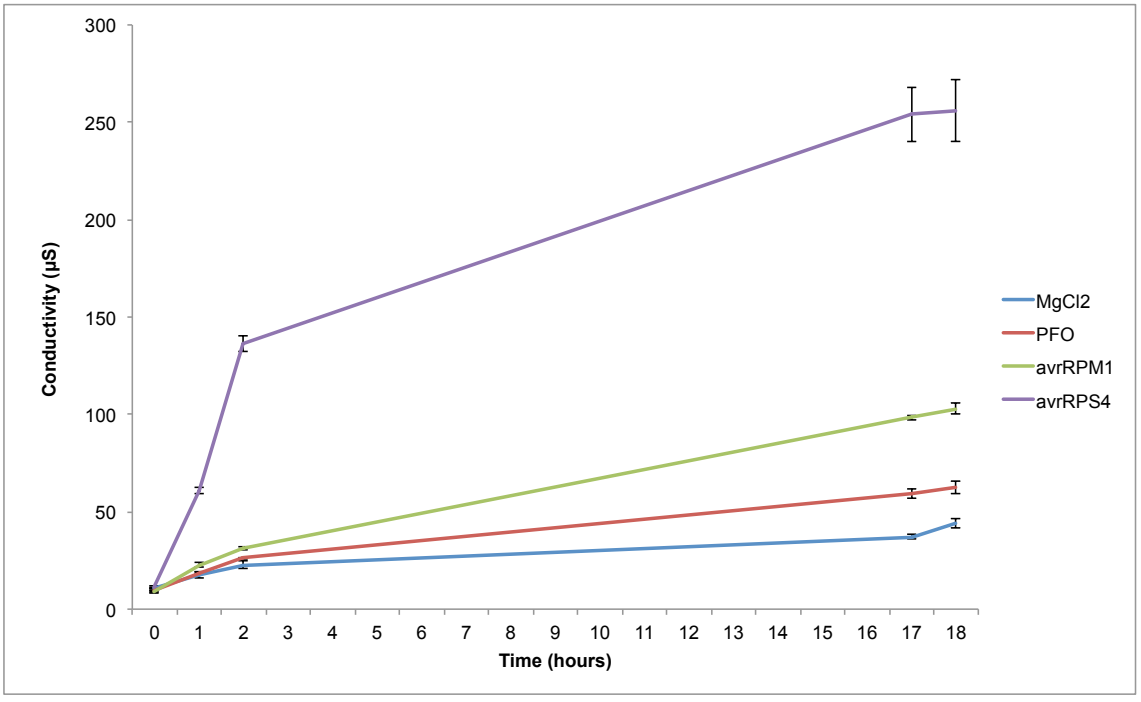
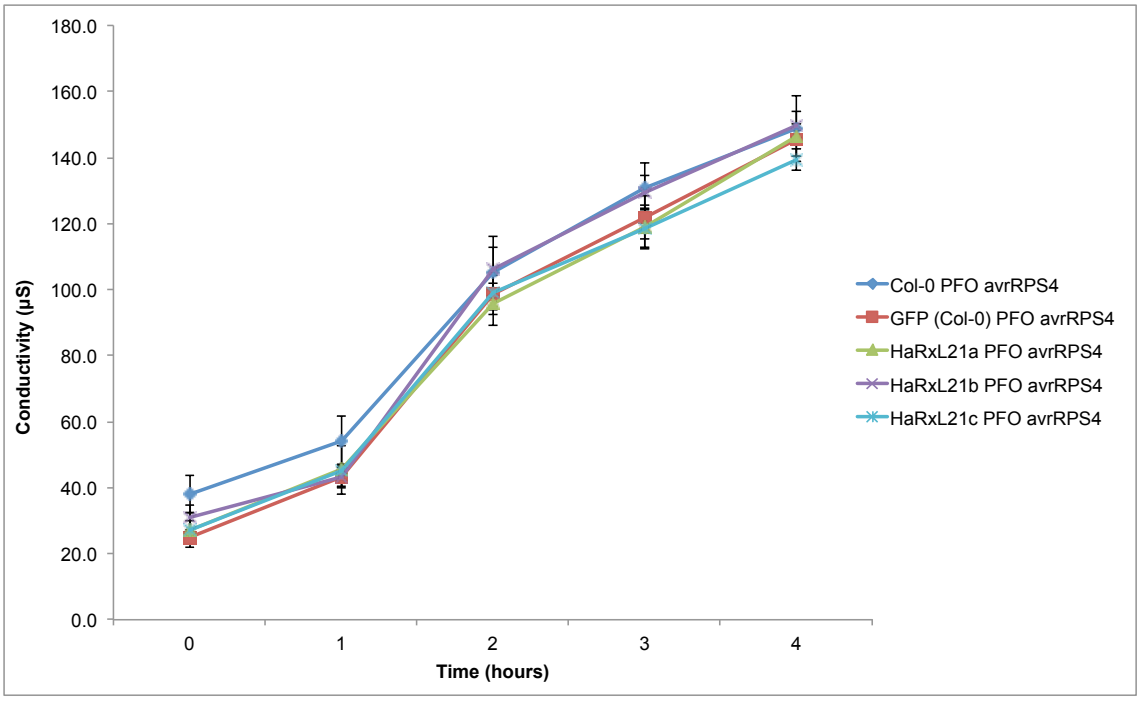


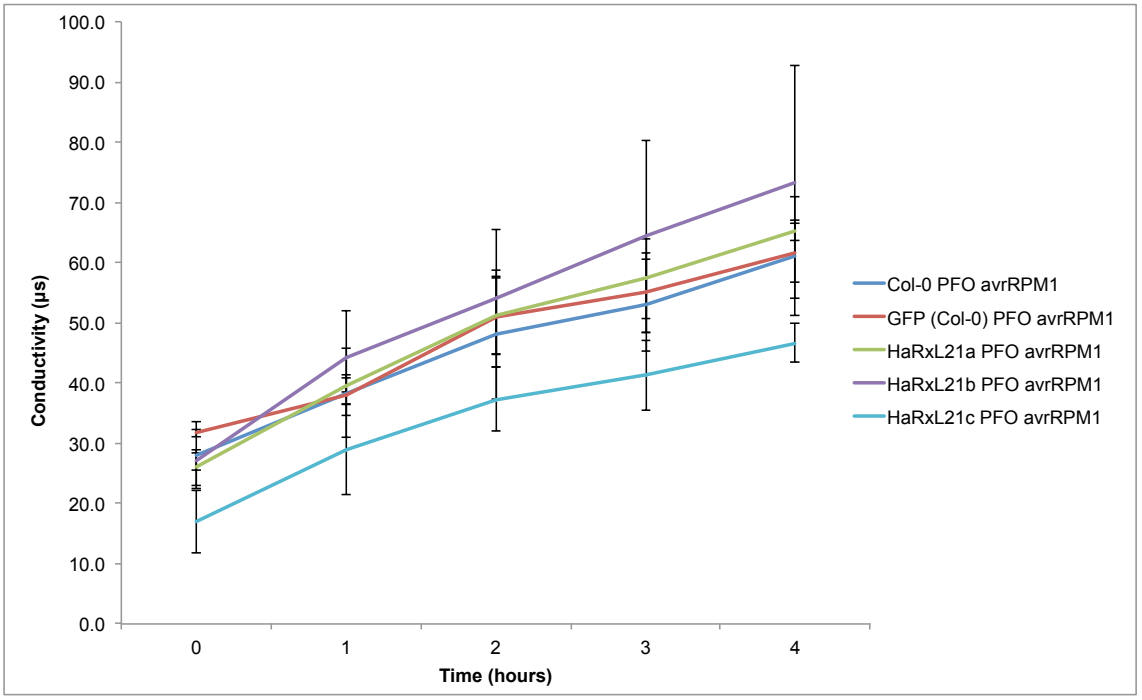
Figure 3.16: *Arabidopsis thaliana* leaf discs show increased ion leakage after infiltration with *Pseudomonas fluorescens* expressing the effector protein AvrRPM1 or AvrRPS4.

Six week old *A. thaliana* plants were infiltrated with *Pseudomonas fluorescens* (*PFO)* +/- the delivery of *Pseudomonas syringae* effectors AvrRPM1 and AvrRPS4 and a $MgCl_2$ control. Leaf discs removed and floated in sterile distilled water, the conductivity of which was measured over time. Error bars display standard error (n=3).

A conductivity experiment was then performed to determine whether the presence of HaRxL21 would suppress ion leakage due to programmed cell death. *A. thaliana* lines HaRxL21a-c, Col-0 and Col-0 35S::GFP lines were infiltrated with *PFO*::AvrRPM1 and *PFO*::AvrRPS4. Plants were also infiltrated with $MgCl_2$, *PFO* as controls to determine whether there was any difference in ion leakage in the absence of AvrRPM1 and AvrRPS4. There was a negligible increase in conductivity observed over time across all plant lines when treated with $MgCl_2$ and *PFO* (data not shown). There was found to be no difference in ion leakage between HaRxL21a and b compared to Col-0 and Col-0 GFP as a response to AvrRPS4 or AvrRPM1 (Figure 3.17). HaRxL21c showed lower conductivity throughout the whole time course, however the rate at which conductivity increased was similar to controls. In addition, since this effect was only observed in 1 of the 3 HaRxL21-expressing lines it is unlikely to be biologically relevant.



(a)



(b)

Figure 3.17: HaRxL21 does not suppress programmed cell death in response to AvrRPS4 or AvrRPM1 as determined by ion leakage measurements.
Ion leakage of 35S::HaRxL21 over-expressing lines in response to delivery of (a) AvrRPS4 or (b) AvrRPM1 by *Pseudomonas fluorescens*, as measured by changes in conductivity over time of a water solution in which bacteria-infiltrated leaf discs were floated. Error bars display standard error (n=3), no significant difference was found using a T-test.

3.8 Discussion

3.8.1 Phenotype

A. thaliana plants expressing HaRxL21 under the 35S promoter from Cauliflower mosaic virus were found to display a round leafed phenotype compared to the GUS protein expressed under the same promoter or to Col-0 wild type plants. In the case of HaRxL21a and to a lesser degree HaRxL21b, stunted growth was also observed (figure 3.1). Comparison of HaRxL21-expressing plants to knock out lines for protein interacting targets found by Mukhtar et al. (2011) has identified that plants with altered expression of TCP14 and TPL exhibit altered leaf morphology. However a large scale screen for leaf morphology phenotypes following mutagenesis was able to isolate hundreds of *A. thaliana* plants which displayed unusual leaf phenotypes (Berná et al., 1999) and it is therefore likely that there are many factors which could cause leaf phenotype to differ from wild type Col-0. It is also possible that sites of insertion of HaRxL21 into the genome may cause this phenotype, although this is unlikely since it is observed in all three independently transformed lines.

The phenotype of these *A. thaliana* lines may therefore give an indication of which interacting targets are important for this effector, since the TCP14 knockout and the TPL over-expressing plants display a similar phenotype to those expressing HaRxL21. However, these interactions need to be verified by yeast-two-hybrid one on one testing and *in planta* verification. It is important to note that during infection, the effector will not be present in the plant at high enough levels, for a long enough period of time, or at at early enough developmental stage to influence development and cause phenotypic changes such as these.

3.8.2 *Hpa* pathogenicity

The findings of Fabro et al. (2011) were that HaRxL21 was shown to enhance susceptibility to *Hpa* isolate Noco2. Since the Noks1 isolate was derived from an oospore of Noco2 (Rehmany et al., 2005), the findings here are in agreement with Fabro et al. (2011), in that when constitutively expressed *in planta*, HaRxL21 is causing enhanced susceptibility to *Hpa* isolate Noks1. Interestingly, this enhanced susceptibility was not found to be isolate specific and enhanced susceptibility was also observed to the isolate Maks9. In both cases, HaRxL21a did not support enhanced growth of *Hpa*, it may be hypothesised that this is because these plants commonly exhibit the smallest growth phenotype (Figure

3.1). When spraying 14 day old *A. thaliana* plants with *Hpa*, it is usual for two true leaves have formed in addition to the cotyledons. The stunted growth phenotype of HaRxL21a results in a smaller leaf surface area for spraying of spores, therefore causing a lower sporangiophore count after infection, meaning that scoring of *Hpa* phenotype in HaRxL21a may show comparable susceptibility.

When the *Hpa* isolate Emco5 was sprayed onto HaRxL21a,b and c, with F1/3, Col-0 and Col-0 GUS controls, a very small number of sporangiophores per seedling were observed across all the *A. thaliana* lines tested which were in a Col-0 background. This was unexpected because Emco5 has been observed to be virulent on Col-0 seedlings (for example in in Yoshioka et al. (2001)). However, it has been shown that Col-0 exhibits developmentally mediated resistance to Emco5, wheras the *A. thaliana* ecotype Wassilewskija does not (McDowell et al., 2005). Therefore the findings here are consistent with this; the only line which does not show a resistance phenotype to Emco5 was Wassilewskija (ws-eds).

In the context of this knowledge, this experiment was therefore asking whether the presence of HaRxL21 compromises the developmentally mediated resistance displayed by Col-0 to Emco5, which was not found to be the case. To determine whether HaRxL21 expression enhances virulence to the *Hpa* isolate Emco5 prior to resistance development, 10 day old HaRxL21-expressing seedlings (too young to exhibit resistance) could be infected with Emco5 and virulence quantified.

3.8.3 *Phytophthora infestans* pathogenicity

It has been shown here that *in planta* expression of HaRxL21 enhances growth of *Hpa* and *B. cinerea*, and it has been previously shown that HaRxL21 enhances *P. syringae* growth (Fabro et al., 2011). It was therefore expected that growth of a pathogen related to *Hpa*, the oomycete *Pi*, would also be enhanced by the presence of HaRxL21.

A. tumefaciens mediated transient expression of HaRxL21 in *Nb* was used to test whether the presence of this effector enhanced growth of *Pi*. The effector delivery method used was the same as in Bos et al. (2010) to study function of the *Pi* effector AVR3a. No difference was observed between *Pi* growth in the presence of HaRxL21 compared to an empty vector control. However, imaging of the biotrophic leading edge of infection using tdTomato RFP was found to be an effective way of monitoring *Pi* growth compared with observing lesion size in white light. This method will have use in future studies of effector function from both *Pi* and *Hpa*.

One explanation for the lack of susceptibility advantage conferred by HaRxL21 to *Pi*

may be due to the effector repertoire of *Pi* and a redundancy in host targets between effectors from *Pi* and *Hpa*. Effectors from *Hpa* and *P. syringae* have been shown to share common targets (Mukhtar et al., 2011) so it is likely that more closely related oomycete pathogens may share host targets. It is possible that the susceptibility advantage provided by HaRxL21 to *Hpa* may be pathogen specific, as unlike the obligate biotroph *Hpa*, there is a necrotrophic phase to the *Pi* infection cycle which may not benefit from the action of HaRxL21. Another explanation may be host specificity, as HaRxL21 may target *A. thaliana* proteins which are not present in *Nb* or do not share similar enough sequence homology for HaRxL21 alteration of their function to be conserved across host species.

3.8.4 *Botrytis cinerea* pathogenicity

SA and JA-dependent responses (which plants utilize to defend themselves against biotrophs and necrotrophs respectively) appear to be antagonistic (Glazebrook, 2001) and it has been suggested that pathogens might modulate the balance between SA and JA responses in their favour (Grant and Jones, 2009). This could be useful to *Hpa*, an obligate biotroph, if it is able to remove of this repression of early JA genes and therefore encourage the plant to concentrate its resources in defending itself against necrotrophic pathogens such as *Botrytis cinerea* (*B. cinerea*). It might therefore be expected that if HaRxL21 alters *A. thaliana* susceptibility to *B. cinerea*, it will be in the direction of reduced susceptibility as the plant is 'primed' to defend itself against necrotrophic pathogens due to manipulation of the antagonistic relationship between the SA and JA responses.

A potential mechanism for this reduction in susceptibility can be defined by studying the model for JA gene regulation by Pauwels et al. (2010), which suggests that early JA genes are repressed by the recruitment of Novel Interactor of Jaz (NINJA) which in turn recruits TPL through direct binding via the EAR motif, therefore bringing about transcriptional repression by histone modification (Pauwels et al., 2010). One of the interacting protein targets of HaRxL21 was found by Mukhtar et al. (2011) to be TPL and the amino acid sequence of HaRxL21 also contains an EAR motif, akin to NINJA. One possible hypothesis, therefore is that HaRxL21 may therefore interfere with this pathway, possibly by titrating TPL away from the complex and therefore removing repression of JA responsive genes.

The *B. cinerea* phenotype observed did not agree with this hypothesis, as we would have expected a reduction in growth of this pathogen. This result is unusual as we might not expect an effector protein from an obligately biotrophic oomycete pathogen to perturb defense against a necrotroph such as *B. cinerea*.

One of the aims here was to establish whether *A. tumefaciens* mediated transient expression in *Nb* could be used to screen for susceptibility advantage by *Hpa* effectors, in particular HaRxL21. This system would allow for rapid screening of mutated versions of the effector to determine functionality of different domains. However, the susceptibility advantage conferred to *B. cinerea* in *A. thaliana* was not replicated in *Nb*. These results, together with the result that transient expression of HaRxL21 does not enhance susceptibility to *Pi*, suggest that maybe the downstream targets of HaRxL21 are not present in *Nb*. This could be investigated further by yeast-2-hybrid (Y2H); determining whether the interacting partners of HaRxL21 have relatives in *Nb* which HaRxL21 is able to interact with.

The results obtained in figure 3.12 suggest that expression vector plays a role in pathogenicity effects of HaRxL21. This may be because the presence of the HA tag is interfering with the effector function, for example localisation, or binding of protein or DNA targets. Using the pGRAB vector for transient expression of HaRxL21 in *Nicotiana benthamiana* and comparing it to either empty pGRAB or GFP in the pGRAB vector might yield results more concurrent with the enhanced *B. cinerea* phenotype observed during *in planta* expression of HaRxL in *A. thaliana*. However the down-side of using pGRAB for transient expression is that it would not allow for detection of protein levels by western blotting (except by specific antibodies to the effector), to ensure that expression continues throughout the infection time course.

3.8.5 *Pseudomonas syringae* pathogenicity

Using the EDV system (Sohn et al., 2007), it was shown that HaRxL21 delivery enhances growth of *Pst lux* compared to *Pst lux* expressing AvrRPS4-AAAA control, but not compared to *Pst lux* alone, 96 hours post infection. However *Pst lux* alone is not a representative control in this case, as it it likely that the process of replicating the EDV6 vector may slow bacterial replication compared to *Pst lux* alone. The variability in this screen is high however, due to the low number of replicates possible within plant growth and time constraints. Fewer time points (for example taking samples 24 h post infection is not necessary) would enable greater replication and increase confidence in these results.

It is also possible that within the effector arsenal of *P. syringae*, there are effectors which function in a similar manner to HaRxL21. Like *Hpa*, *P. syringae* is a biotrophic pathogen and therefore similar host targets would benefit both pathogens. The soil bacterium *Pseudomonas flurescens* has been engineered to express the type III secretion machinery from *Pseudomonas syringae* pv. *syringae* 61 (Thomas et al., 2009). This system would allow

the virulence of HaRxL21 to be assayed without possible interference or overlap from *P. syringae* effectors, and should be used for any future screens of this kind.

3.8.6 ETI disruption by HaRxL21?

To establish whether HaRxL21 is able disrupt the process of effector triggered immunity, it was investigated whether *in planta* expression of HaRxL21 compromises *Hpa* resistance phenotypes, and is able to prevent ion leakage caused by the hypersensitive response in response to the delivery of *P. syringae* avirulence genes.

The *A. thaliana* ecotype Col-0 is resistant to *Hpa* isolates Emoy2 and Hiks1 due to the recognition of ATR4 by the resistance protein RPP4, and ATR7 by RPP7 respectively (van der Biezen et al., 2002). Resistance against these *Hpa* isolates was found to not be compromised by the expression of HaRxL21 in a Col-0 background (figures 3.14 and 3.15). These data suggest that HaRxL21 does not function to disrupt these examples of ETI in *A. thaliana*.

When an avirulence gene is recognised by the corresponding resistance protein (for example the recognition of AvrRPM1 by RPM1), this is frequently associated with programmed cell death known as the hypersensitive response (Mur et al., 2008). To determine whether this was compromised by the presence of HaRxL21, ion leakage due to recognition of avirulence genes was measured. Measurement of conductivity changes was used by Jamir et al. (2004) to identify *P. syringae* effectors which were able to suppress programmed cell death in plants. This system was therefore chosen and applied to *A. thaliana* plants expressing HaRxL21, it was found that *in planta* expression of HaRxL21 here was not able to suppress ion leakage due to recognition of these *P. syringae* effector proteins.

As HaRxL21 did not seem to compromise ETI but did enhance susceptibility to pathogens, its role is likely to be in suppression of PTI. It is therefore logical to concentrate on the underlying mechanism behind susceptibility advantages conferred by HaRxL21 during virulent plant-pathogen interactions.

Chapter 4

Protein-Protein Interactions of HaRxL21

4.1 Introduction

The yeast-two-hybrid (Y2H) system was first developed by Fields and Song (1989), and utilises the GAL4 protein of the yeast *Saccharomyces cerevisiae*. The GAL4 protein is a transcriptional activator which when yeast is grown on galactose media, activates expression of genes required for galactose utilisation. This system is so called 'two-hybrid' because it involves two hybrid proteins, the N-terminal domain of GAL4 which is required for DNA binding (DB), and the C-terminal domain which is required for transcriptional activation (activation domain; AD). These domains can be separated and each fused to a protein of interest, being reconstituted to an active form by the interaction of these proteins (Fields and Song, 1989).

Using Y2H, interactions were tested between pathogen effectors from *P. syringae* and *Hpa* with ~8000 open reading frames from predicted protein-encoding genes in *A. thaliana* (Mukhtar et al., 2011). In this study, HaRxL21 was found to interact with four proteins; AT1G15750 (TOPLESS), AT3G07780 (OBE1), AT3G47620 (TCP14) and AT5G55100 (SWAP). A matrix-two-hybrid approach (as described by Häuser et al. (2012)) was used for this study and is ideal as it allows large scale screening for interacting proteins against a library. However it is important to validate interactions and determine whether observed interactions actually occur *in planta*. These interactions will therefore be validated by in house repetition of Y2H screening and by *in planta* methods such as Bimolecular fluorescence complementation (BIFC) and Co-Immunoprecipitation (Co-IP), both of which require proteins to be co-located as well as physically able to interact.

4.1.1 TCP14

Teosinte Branched Cycloidea and PCF (TCP) genes are plant-specific transcription factors which have a bHLH motif, facilitating DNA binding and protein-protein interactions (Martín-Trillo and Cubas, 2010). There are 24 TCP genes in the *A. thaliana* genome (Cubas, 2002), loss-of-function mutants of many of which cause developmental defects (Martín-Trillo and Cubas, 2010).

A role for TCP14 in pathogenicity has been suggested by the literature; differential regulation of TCP14 was observed during *A. thaliana* clubroot infection caused by *Plasmodiophora brassicae* (Siemens et al., 2006) and during infection of *A. thaliana* by cabbage leaf curl virus (CaLCuV) infection (Ascencio-Ibáñez et al., 2008). Furthermore, the importance of TCP14 in plant disease has been suggested by Mukhtar et al. (2011), who found that TCP14 was targeted by proteins from both *Hpa* and *P. syringae* a total of 29 times, including HaRxL21. TCP14 was designated a 'hub' protein in the *A. thaliana* immune network due to the large number of interactions with both pathogen effectors and *A. thaliana* proteins involved in defense. These interacting partners include transcription factors, proteins involved in RedOx maintenance and proteins involved in hormone signalling (figure 4.1) (Mukhtar et al., 2011).

4.1.2 SWAP

The *Drosophila melanogaster* Suppressor-of-White-APricot (DmSWAP) protein regulates splicing of the *white-apricot* allele of the *white* gene (Bingham et al., 1988) and has been defined as an alternative splicing regulator. Mouse (MmSWAP) and human (HsSWAP) homologues have also been described (Denhez and Lafyatis, 1994).

There has been very little characterisation of *A. thaliana* SWAP (encoded by AT5G55100), although during screens of T-DNA insertion lines for AT5G55100, Luhua et al. (2013) found that these lines showed increased tolerance to Osmotic stress and ABA stress. SWAP has also been identified during screening for SUMOlation targets (Meier, 2012). The role of SWAP in plant defense against pathogens is little studied, and no changes in *SWAP* expression can be observed under biotic stress conditions using the *A. thaliana* eFP browser (Winter et al., 2007).

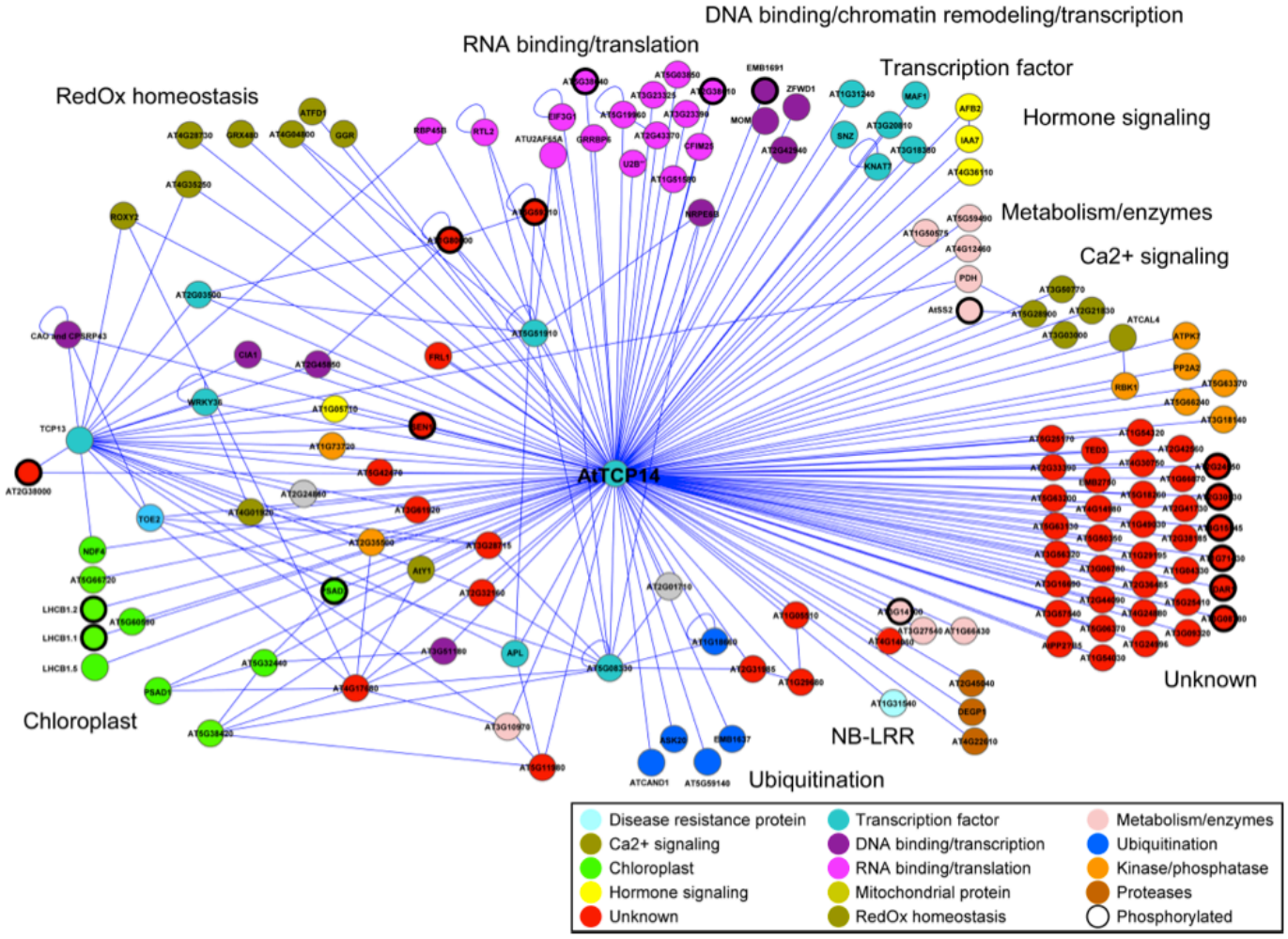


Figure 4.1: The TCP14 interactome in *A. thaliana*.

TCP14 was found by Y2H to interact with a large number of proteins in the *A. thaliana* immune network which encompass many pathways. Reproduced with permission from (Mukhtar et al., 2011).

4.1.3 OBE1

The *A. thaliana* gene *OBERON1* (*OBE1*) (AT3G07780) encodes a protein with a plant homeodomain (PHD) finger domain, and a coiled-coil domain. OBE1 functions redundantly with the related protein, OBE2 during development of the shoot and root apical meristems (Saiga et al., 2008). A potential role for OBE1 in stress responses has been implicated by the predicted targeting of *OBE1* messenger RNA (mRNA) by the stress-regulated MicroRNA (miRNA) *MIR403* in *A. thaliana* (Sunkar and Zhu, 2004).

The role of OBE1 in plant disease resistance is not well documented, and no significant differential expression upon biotic challenge can be observed using the *A. thaliana* eFP browser (Winter et al., 2007). However, OBE1 was formerly known as PVIP2 (*Potyvirus* VPg-interacting protein), the PVIP protein family are such named because they are targeted by virus genome-linked proteins (VPgs) of a range of potyviruses. These proteins have been found to support virus movement throughout the plant and reducing expression levels of *PVIP* genes has been found to reduce susceptibility to TuMV (Dunoyer et al., 2004). A role in *Hpa* pathology has also been suggested; targeting of OBE1 by pathogen effector proteins was identified using Y2H by Mukhtar et al. (2011). During verification of effector targets, knocking out OBE1 was found to cause reduced susceptibility to *Hpa* isolate Noco2, suggesting that in this case, effector targets are required for full pathogen virulence (Mukhtar et al., 2011).

4.1.4 TOPLESS

The *A. thaliana* protein TOPLESS (TPL) (AT1G15750) was identified after isolation of a mutant *tpl-1* from the *A. thaliana* ecotype Landsberg erecta (Ler) (Long et al., 2002). This mutant was designated 'Topless' after it showed temperature sensitive transformation of the shoot apical meristem into root, as expression of genes associated with the basal half of an *A. thaliana* embryo were extended into the top. *TPL* encodes a 124 kDa protein, with a predicted C-terminal to LisH (lissencephaly type 1like homology) (CTLH) domain at the N-terminus which is thought to be important for protein-protein interactions or self-dimerization (Emes and Ponting, 2001; Zeng et al., 2006).

TPL was found to be involved in auxin-dependent transcriptional regulation, as Y2H screening resulted in the identification of multiple AUX/IAAs (transcriptional regulators which degrade during the auxin response (Worley et al., 2000)) with TPL. These interactions were also confirmed *in planta* using co-immunoprecipitation from lysate of *A. thaliana* plants with 6xHA fused to TPL (Szemenyei et al., 2008). It has been pre-

viously shown that Aux/IAA proteins contain an Ethylene-responsive element binding factor-associated Amphiphilic Repression (EAR) motif (LxLxL) which is important for mediating transcriptional repression (Tiwari et al., 2004). Mutations to this motif were found to severely weaken interaction the interaction between IAA12/BDL and TPL (Szemenyei et al., 2008). The CTLH domain of TPL has also been shown to be necessary and sufficient for this interaction (figure 4.2) (Szemenyei et al., 2008). These data are particularly interesting in the context of HaRxL21 which also contains an EAR motif. It is therefore a reasonable hypothesis that the interaction of HaRxL21 with TPL is mediated by the EAR motif at the c-terminal of TPL, with the CTLH domain which occurs near the N-terminus of TPL.

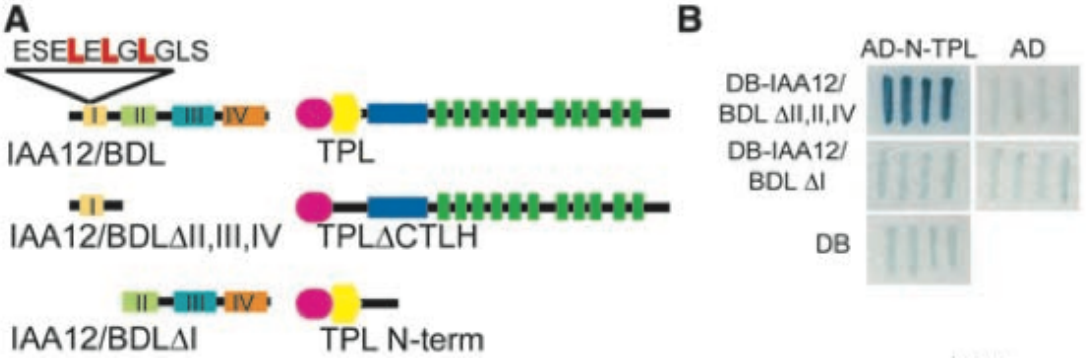


Figure 4.2: The CTLH domain of TPL is necessary and sufficient for interaction with the EAR domain of IAA.
(A) Constructs used for yeast-two-hybrid (Y2H); domain I of IAA12/BDL contains the EAR motif and TPL-N-term contains the CTLH domain. (B) Y2H results showing interaction of AD-N-TPL with BD-IAA12/BDL. Interaction is abolished in the absence of IAA12/BDL domain I. Reproduced with permission from (Szemenyei et al., 2008).

TPL is in a family with four related proteins (TPL-related, known as TPR proteins) (Long et al., 2006) the most similar of which is TPR1, which shares 92% sequence identity and 95% similarity at the amino acid level. The most closely related to TPL and TPR1 is TPR4 which shares 69% sequence identity and 81% similarity to TPL at the amino acid level (Zhu et al., 2010). TPL and it's homologues function redundantly in plant defense; knocking out TPL, TPR1 and TPR4 has been found to incrementally increase susceptibility to *Pseudomonas syringae* (Figure 4.3). In addition, TPR1 has been implicated in R-gene mediated resistance (Zhu et al., 2010).

TPL is an interesting target for a pathogen effector protein since it has found to interact with several key regulators of hormone pathways known to be involved in plant defense against pathogens (Arabidopsis Interactome Mapping Consortium, 2011). For example, interactions have been found with NIMIN2 and NIMIN3 which are regulators of SA signalling, with ERF9 which is a transcriptional regulator of ET signalling and interaction

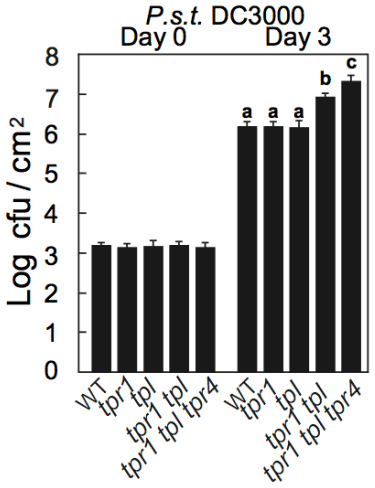


Figure 4.3: Knocking out TPL, TPR1 and TPR4 causes enhanced susceptibility to *P. syringae*.
TPL, TPR1 and TPR4 function redundantly in plant defense against *P. syringae*, shown by colony counts 3 days post infection. Reproduced with permission from Zhu et al. (2010).

with the co-repressor NINJA or directly with JAZ5 or JAZ8 suggests a role in regulation of JA signalling (Arabidopsis Interactome Mapping Consortium, 2011). These interactions suggest a role for TPL as a 'master regulator' and therefore it is an important target if pathogens are able to manipulate its mode of action.

More recently, high throughput Y2H has been used to identify the interacting targets of TPL and TPR proteins and produce a 'TPL interactome', which is graphically represented in figure 4.4 (Causier et al., 2012). TPL and TPR proteins were found to be involved in a wide range of biological processes, notably in developmental pathways, hormone signalling pathways and response to stress. Interestingly, in addition to the EAR motif LxLxL, all of the previously characterised repression domains (DLNxxP, R/KLFGV and TLxLF (Ohta et al., 2001; Matsui et al., 2008; Ikeda and Ohme-Takagi, 2009; Causier et al., 2012)) were enriched amongst transcription factors found to interact with TPL and TPR proteins. These data support the role of TPL/TPRs as 'master regulators' or general repressors in *A. thaliana*.

Evolutionary conservation of TPL-mediated repression has also been found to be conserved between mosses and angiosperms, since TPL and TPR2 from the moss *Physcomitrella patens* were able to interact with moss AUX/IAA proteins which contain the domain LxLxPP (Causier et al., 2012).

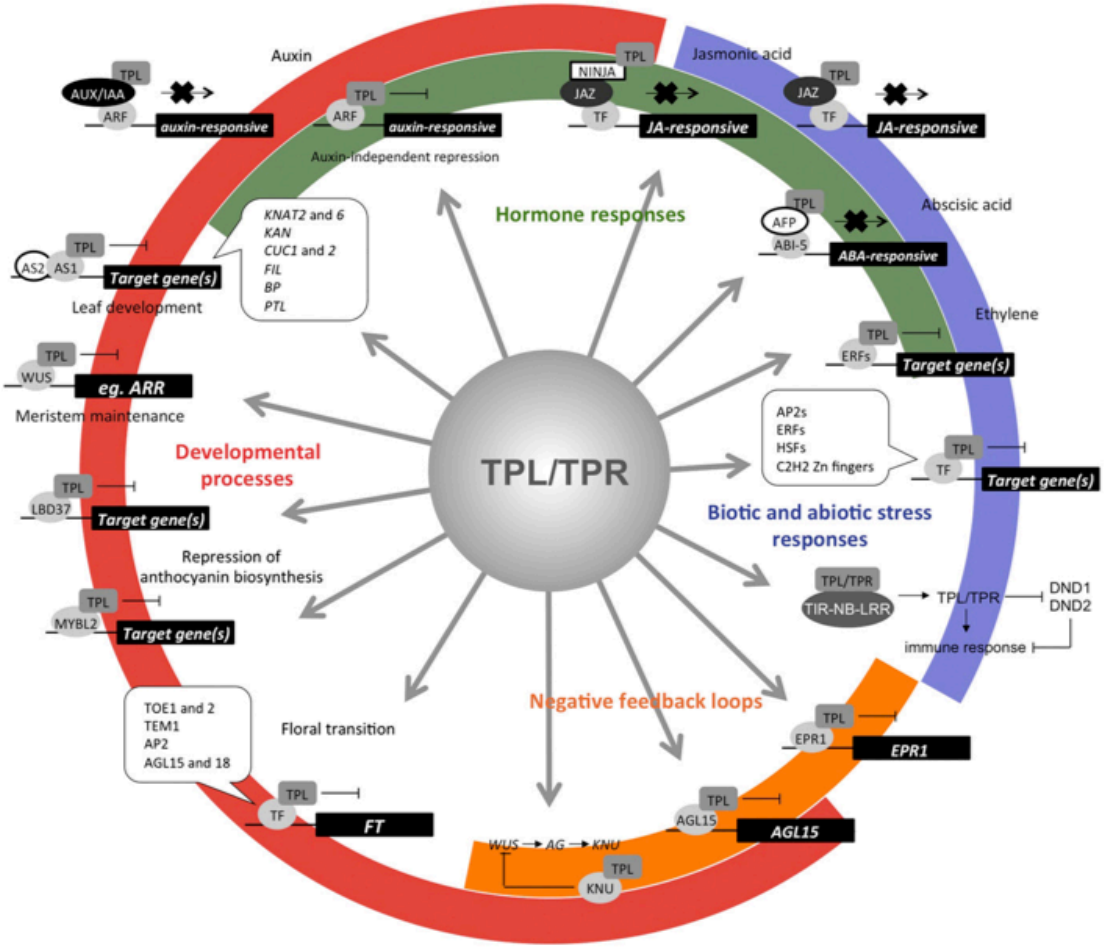


Figure 4.4: The TOPLESS Interactome

Biological processes in which TPL and TPRs have been found to interact. Colored arcs represent the different processes in which the TPL family may act, of note in the context of plant-pathogen interactions are green (hormone responses) and blue (biotic and abiotic stress responses). Reproduced with permission from Causier et al. (2012).

4.1.5 Aims

The aims of the work described in this chapter were:

1. To verify protein-protein interactions of HaRxL21 with TCP14, OBE1, SWAP and TPL using Y2H.
2. For confirmed interactions, identify the interacting domains and specific amino acids involved.
3. To validate protein targets of HaRxL21 *in planta*.
4. To examine the impact of these interactions on pathogenicity phenotype; determining whether knocking out targets alters resistance to pathogens and whether preventing interaction also prevents the effector from providing a susceptibility advantage.

4.2 Validation of HaRxL21 Protein Targets

To validate interacting targets of HaRxL21 identified by Mukhtar et al. (2011), binary Y2H testing was performed to determine whether HaRxL21 interacted with TCP14, OBE1, SWAP and TPL (following the protocol described by Dreze et al. (2010)). Screens were performed in both directions; with the effector and the target *A. thaliana* protein fused to both the activation domain and the DNA binding domain. In all cases, yeast growth on plates lacking leucine (Leu) and tryptophan (Trp) was monitored to confirm that cells are diploid and have taken up the plasmids AD-X (selectable on -Trp) and DB-X (selectable on -Leu).

The reporter system used here is described in Dreze et al. (2010) and uses two reporter genes for protein-protein interaction; Histidine (His) and Adenine (Ade). Growth on plates lacking His or Ade is therefore an indicator that the AD-X and DB-X proteins are interacting with each other. 3-amino-1,2,4- triazole (3AT), a competitive inhibitor of the HIS3 gene product is also added to selective media to add confidence that the reporter gene product is being produced by inhibiting growth due to base line activation. Using different concentrations of 3AT can also give an indication of interaction strength. An artefact of the Y2H system is autoactivation. Autoactivating proteins are able to activate transcription in the absence of the C-terminal GAL4 activation domain, meaning that yeast is able to grow on the reporter plates (-His) without the occurrence of protein-protein interaction. To control for this, screening is performed to check for proteins which exhibit autoactivation. This is done by mating DB-X constructs with yeast harbouring empty pAD and removal of constructs which are able to grow on selective media (-His).

The interaction of TCP14 with HaRxL21 was investigated. Yeast growth was observed on -Leu -Trp plates, indicating that mating of the two strains had occurred successfully. There was no growth when TCP14 was screened against empty pAD or empty pDB, showing that no auto-activation occurred. TCP14 was found to not interact with HaRxL21 in either direction, as no growth was observed on the -His plates (figure 4.5a). The screen was repeated and there was found to be slight yeast growth when TCP14 was screened against empty pDB, suggesting that it may show slight DNA binding activity.

The presence of SWAP was found to cause auto activation; yeast grew on the reporter (-His) plate when yeast expressing DB::SWAP was mated with the empty pAD vector. This meant that the interaction with HaRxL21 was only able to be examined in one direction; DB::HaRxL21 with AD::SWAP and an interaction between these proteins was found (figure 4.5b).

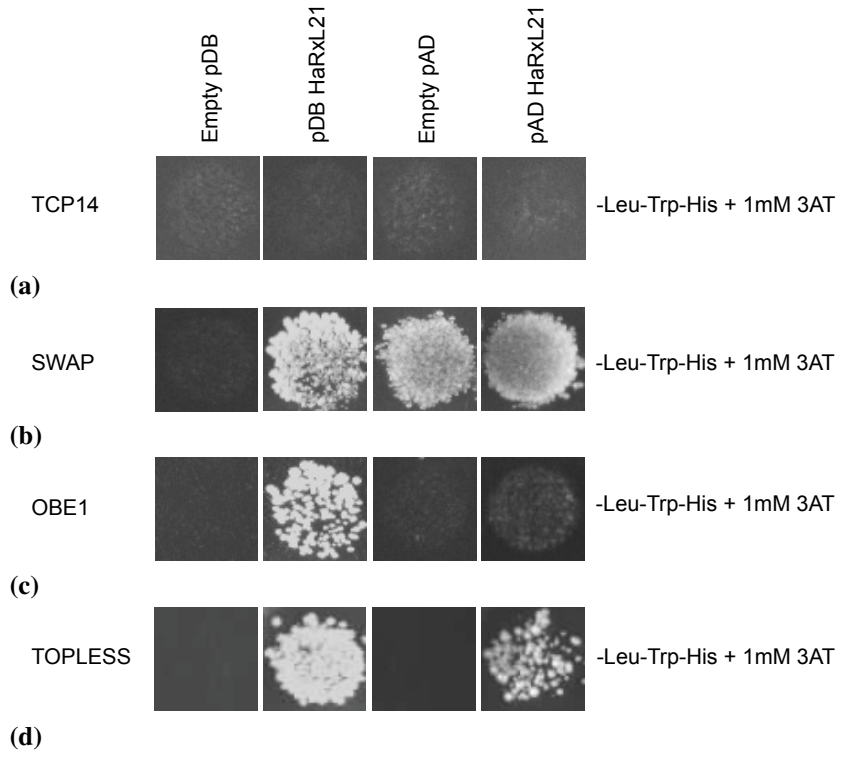


Figure 4.5: Validation of HaRxL21 Protein Targets by Y2H.
The interaction of HaRxL21 with TCP14, SWAP, OBE1 and TPL, as shown by yeast growth on media lacking Leu, Trp and His +1mM 3AT. Empty vector controls were included to show the presence or absence of auto-activation. Mating of yeast expressing HaRxL21 and TCP14, SWAP, OBE1 and TPL was in all cases confirmed by growth on media lacking Leu and His (data not shown).

The interaction of HaRxL21 with OBE1 was also confirmed and no autoactivation was observed (figure 4.5c).The interaction with OBE1 could only be observed when OBE1 was fused to the activation domain but not in the other direction. The lack of interaction between AD::HaRxL21 and DB::OBE1 was not due to lack of mating as yeast containing these constructs grew on the -Leu -Trp plates.

TPL was found show no autoactivation and to interact by Y2H with HaRxL21 (figure 4.5d). This interaction was observed in both directions although was observed to be stronger when TPL was fused to the activation domain.

4.3 Further characterisation of interactions

4.3.1 The C-terminal EAR motif of HaRxL21 Interacts with the CTLH domain of TPL

Previous work has shown TPL to interact with IAA12 specifically via the interaction of the LxLxL domain of IAA12 and the CTLH domain of TPL (figure 4.2) (Szemenyei et al., 2008). The interaction of HaRxL21 with TPL was therefore further characterised to establish whether HaRxL21 interacts with TPL by the same mechanism. Truncated forms of the effector were cloned, using two reverse primers to determine whether truncation immediately before the EAR motif was sufficient to prevent interaction. Constructs lacking the RxLR-DEER were also generated to determine whether this motif was involved in mediating the interaction. A schematic of truncated forms of HaRxL21 used are shown in figure 4.6. The interaction of HaRxL21 and TPL has been found to be specific to the LxLxL domain at the C terminus of HaRxL21, as deletion of this domain results in no interaction. The interaction was detected in both directions (with TPL fused to both the activation domain and DNA binding domain in yeast), although found to be stronger when TPL was fused to the activation domain, shown by the induction of the Adenine reporter gene and growth on plates lacking Ade, Leu, Trp and His (figure 4.6).

The same HaRxL21 constructs were then tested for interaction with SWAP and OBE1. In both cases, DB::HaRxL21 was tested, since DB::SWAP was found to auto-activate and DB::OBE1 did not interact with AD::HaRxL21. In both cases, neither removal of the RxLR-DEER or the EAR motif from HaRxL21 prevented the interaction from occurring.

To test whether the CTLH domain near the N-terminus of TPL mediated the interaction with HaRxL21, TPLΔCTLH was kindly provided by Szemenyei et al. (2008). The interaction between HaRxL21 and the CTLH domain of TPL was then tested by Y2H in both directions. There was found to be no interaction between HaRxL21 and TPLΔCTLH, shown by the absence of growth on media lacking His. This result indicates that the CTLH domain is where the interaction occurs (figure 4.7). Growth is shown on -Leu -Trp plates to show that mating has taken place and the lack of growth on the -Leu -Trp -His plates is due to removal of interaction.

To prove that removal of the EAR motif did not alter the stability or the localisation of HaRxL21, it was cloned into a Gateway vector fused to an N terminal GFP tag, transformed into *A. tumefaciens* and infiltrated into *Nb* plants. Two days after infiltration, leaves were infiltrated with 4',6-diamidino-2-phenylindole (DAPI), a DNA-specific fluorescent marker (Kapuscinski, 1995) and leaf discs were visualised using a Zeiss confocal

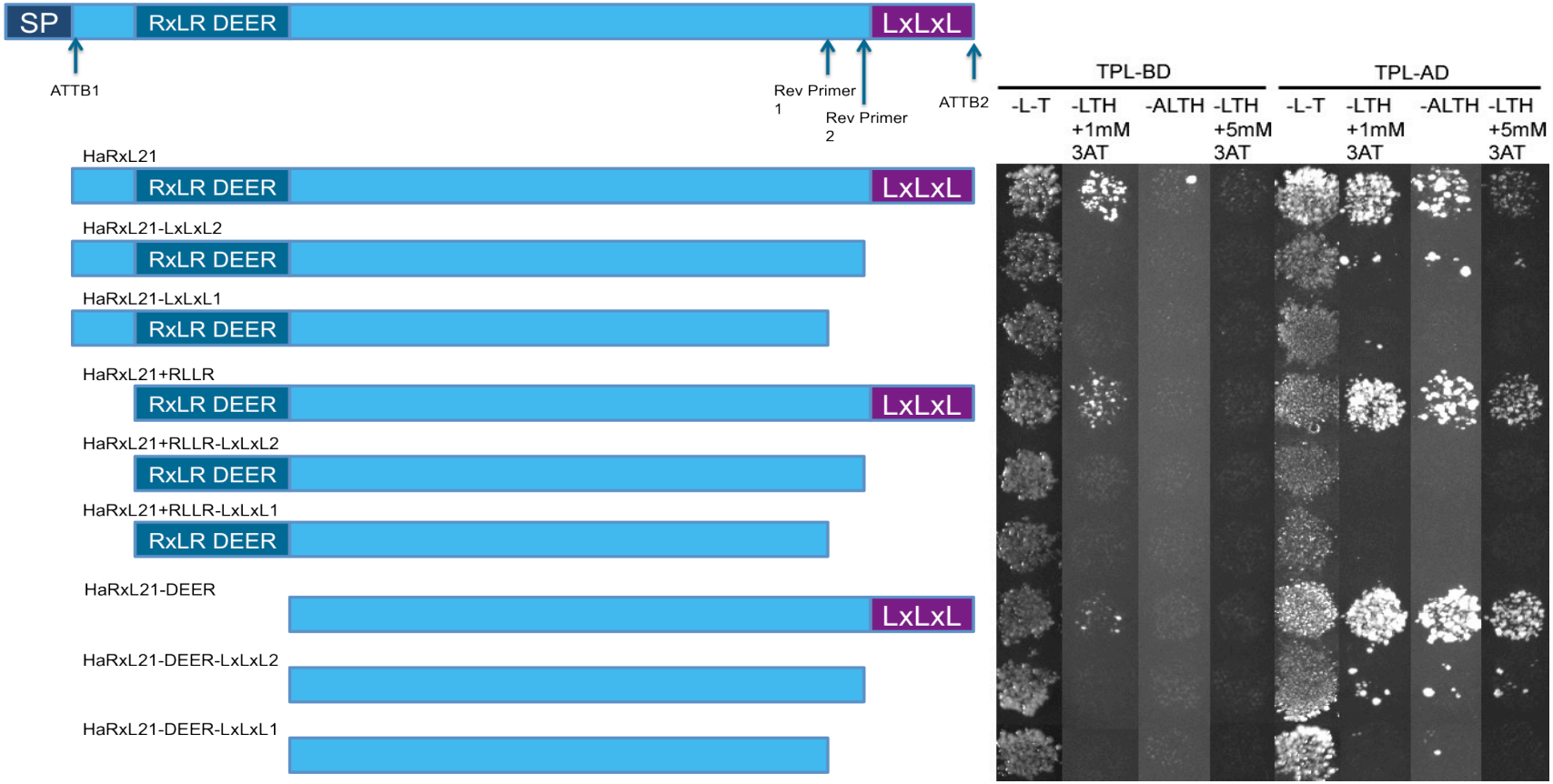


Figure 4.6: HaRxL21 Interacts with TPL by Y2H via the EAR (LxLxL) domain at the C-terminus.

Various constructs of HaRxL21 were tested for interaction with full length TPL. AttB1 and AttB2 denote sites for Gateway cloning into Y2H vectors. Yeast growth on the -Leu-Trp plates confirms that cells are diploid and have taken up the plasmids AD-X (selectable on -T) and DB-X (selectable on -L). The reporter system uses two reporter genes for protein-protein interaction; histidine (-LTH) and Adenine (-ALTH). 3-amino-1,2,4- triazole (3AT) is a competitive inhibitor of the HIS3 gene product and is used to remove weak or background interactions.

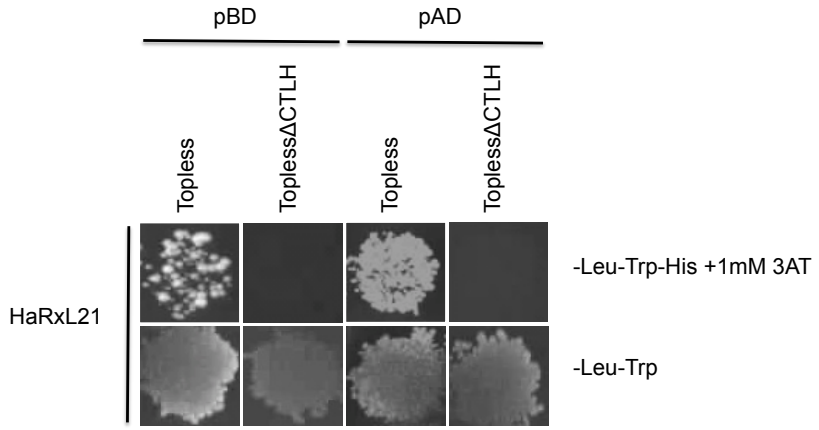
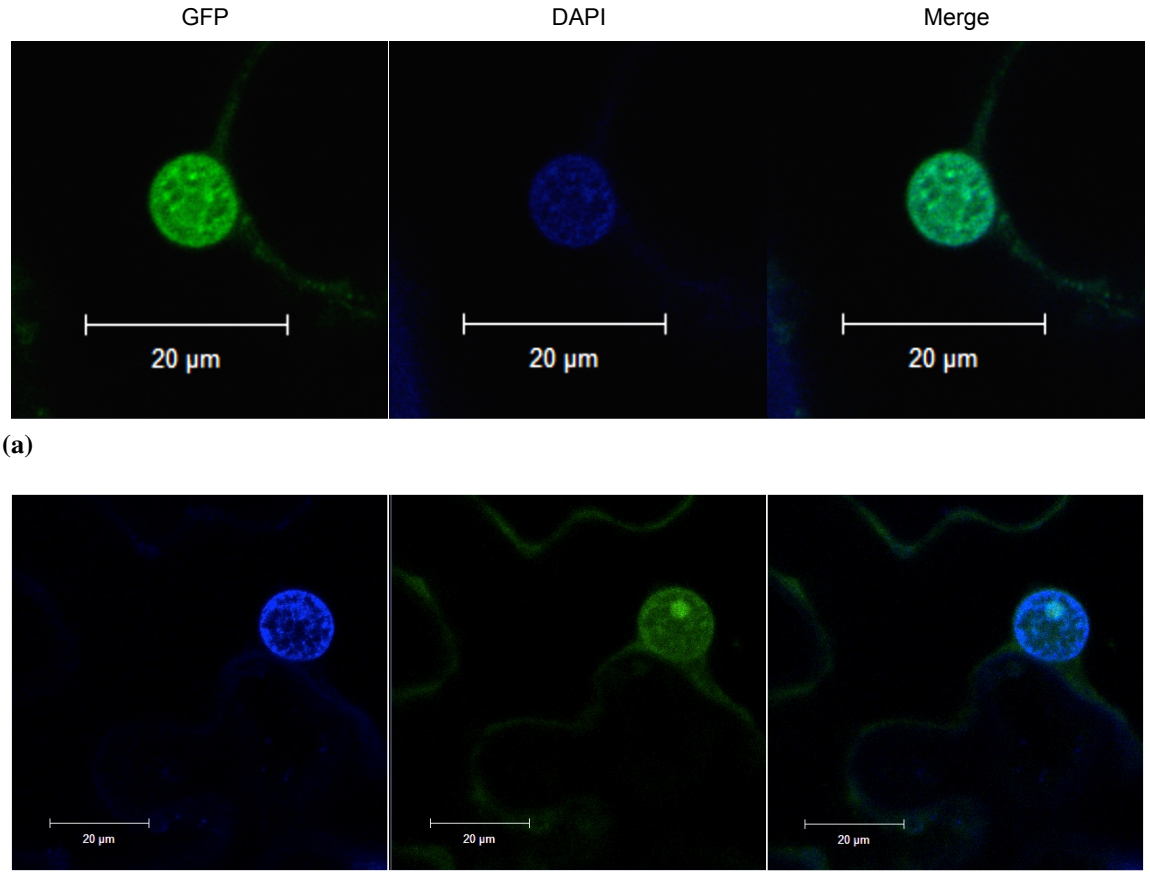


Figure 4.7: HaRxL21 Interacts with the CTLH domain of TPL.
The interaction between HaRxL21 and Tpl or TPLΔCTLH. Yeast growth on the -Leu-Trp plates confirms that cells are diploid and have taken up the plasmids AD-X (selectable on -T) and DB-X (selectable on -L). Growth on plates lacking histidine (-Leu -Trp -His) supplemented with 3AT indicates that the proteins are interacting.

microscope. HaRxL21 was found to be nuclear localised (shown by co-localisation with DAPI), in addition to some membrane localisation (figure 4.8a). The same nuclear localisation was observed for the truncated version (HaRxL21ΔLxLxL) of the effector (figure 4.8b).



(a)

(b)

Figure 4.8: HaRxL21 and HaRxL21ΔLxLxL both localise to the nucleus.
In planta localisations after *Agrobacterium tumefaciens* mediated transient expression in *Nicotiana benthamiana* of (a) GFP::HaRxL21 (b) GFP::HaRxL21ΔLxLxL. GFP is shown in green, nuclear localisation is shown by co-localisation with DAPI, a DNA-specific marker (blue).

4.3.2 HaRxL21 Cala and Noks1 alleles

Alleles of HaRxL21 in the *Hpa* iolates Cala2 and Noks1 were cloned previously into a Gateway entry vector by Sarah Bailey. The Noks1 allele is truncated due to a C to G substitution at position 590 which causes insertion of a stop codon, therefore it does not have an EAR motif at the C-terminus and would be expected to not interact with TPL.

The amino acid sequences of HaRxL21 Emoy2 and Cala2 were aligned using T-Coffee multiple sequence alignment program (Notredame et al., 2000). Alignments are shown in Figure 4.9 and it can be observed that there are 18 amino acid substitutions between the HaRxL21 allele from Emoy2 (upon which this work has been based) and the HaRxL21 allele from Cala2. It is noteworthy that the RXLR-DEER region is conserved, as is the C-terminus of the protein containing the EAR motif.

The Noks1 and Cala2 alleles were cloned into pAD and pDB, and both tested by Y2H against all the original interacting targets of HaRxL21; TCP14, OBE1, SWAP and TPL. No interaction was found to occur between either allele with any of these proteins.

The Phyre^2 (Protein Homology/analogY Recognition Engine V 2.0) (Kelley and Sternberg, 2009) was used to predict the protein structures of HaRxL21 (Emoy2) and HaRxL21 Cala2. It can be observed that protein structure prediction from the amino acid sequences of HaRxL21 Emoy2 and Cala2 differs between these two alleles (Figure 4.10). The EAR motif which mediates interaction with TPL is at the C-terminal of the effectors (shown in red in figure 4.10). The position of the C-terminus of the protein can be observed to be different between the two alleles, interestingly however, it is predicted to be more exposed on the Cala2 allele, indicating that there is potentially nothing blocking this domain from interacting with TPL.

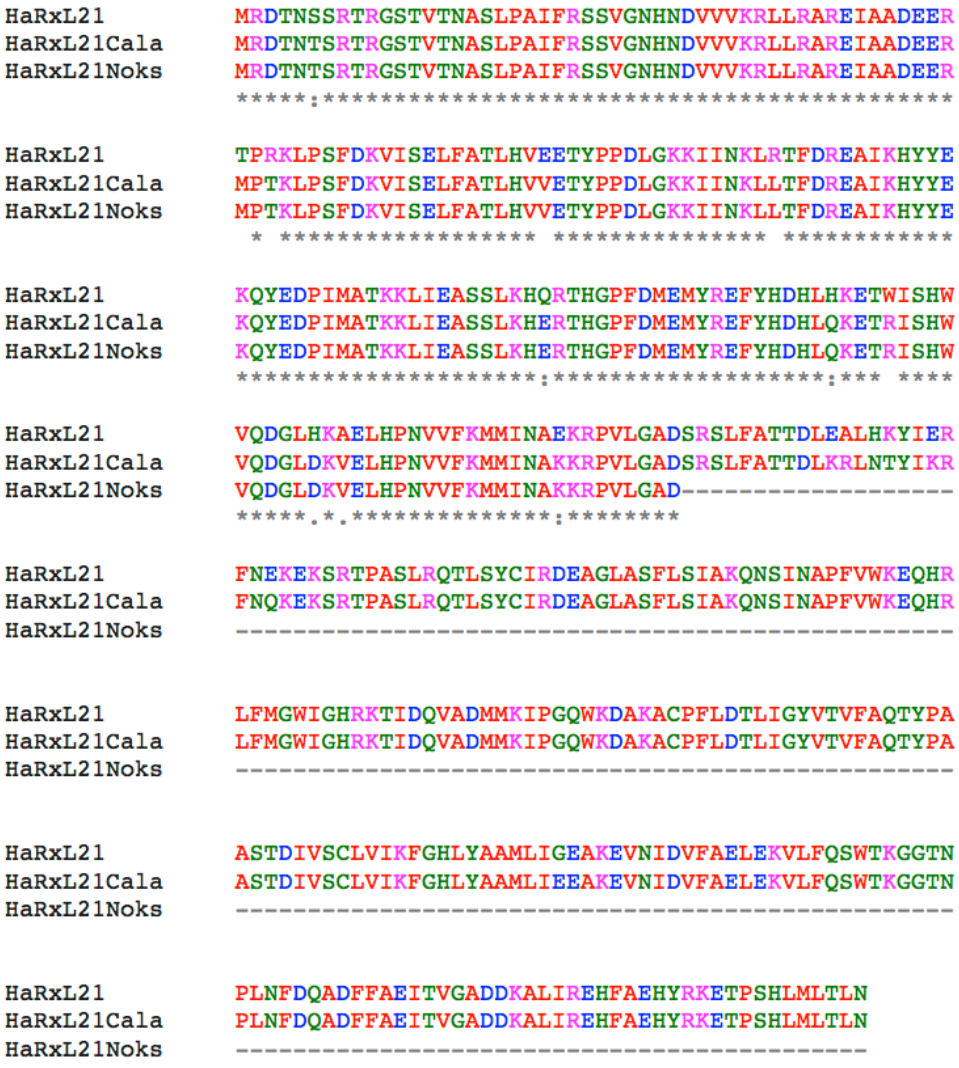
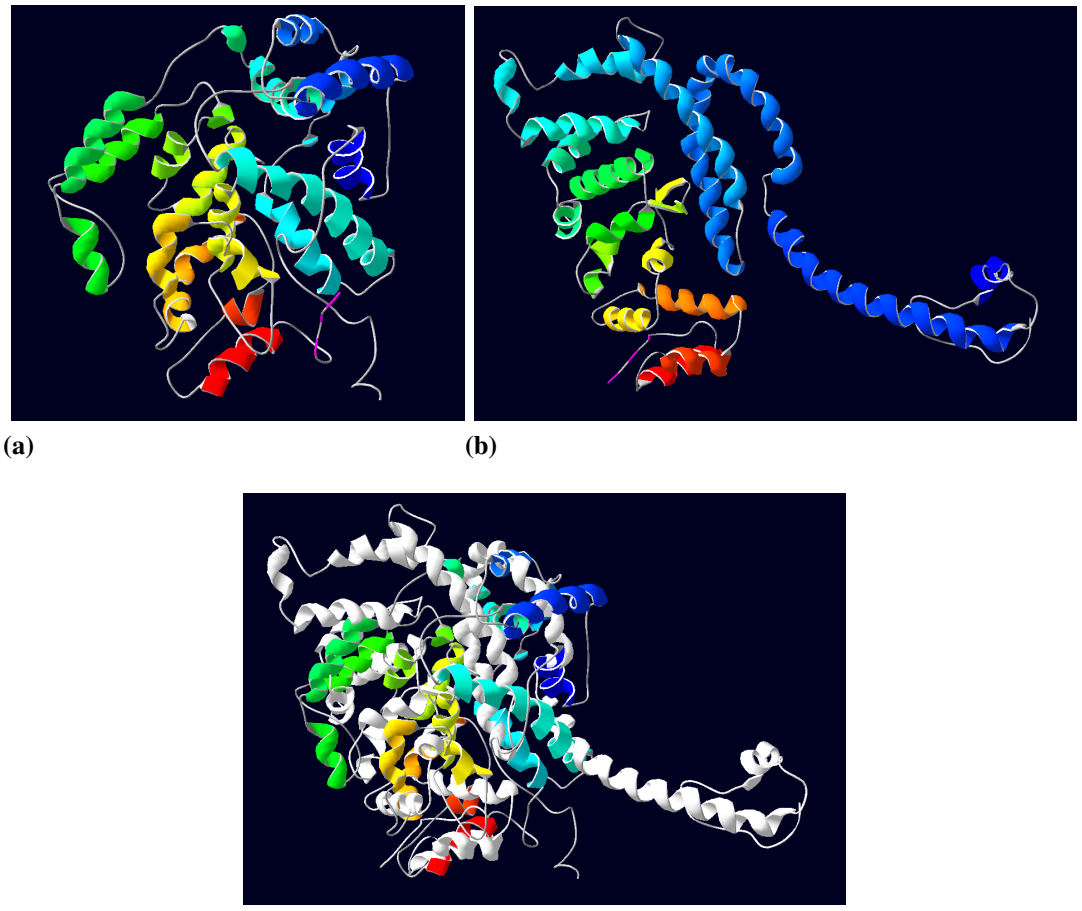


Figure 4.9: Protein sequence alignment between HaRxL21 Emoy2, Cala2 and Noks1 alleles.

Protein sequence alignment between HaRxL21 Emoy2, Cala2 and Noks1 alleles using T-Coffee multiple sequence alignment program (http://www.ebi.ac.uk/Tools/msa/tcoffee/).



(a) **(b)**

(c)

Figure 4.10: HaRxL21 alleles from Emoy2 and Cala2 have different predicted protein structures.

Predicted protein structure of (a) HaRxL21 Emoy2 (b) HaRxL21 Cala2 using the Phyre^2 web tool (Kelley and Sternberg, 2009). The N-terminus of the protein is shown in blue and the C-terminus of the protein is shown in red. Leucine residues which form the EAR motif are shown in magenta. (c) Overlay of HaRxL21 Emoy2 and Cala2 alleles using the Swiss-PdbViewer magic fit tool (http://spdbv.vital-it.ch), showing dissimilarity between the two structures. HaRxL21 Emoy2 is shown in rainbow and HaRxL21 Cala2 is shown in white.

4.3.3 *Solanum tuberosum* and *Nicotiana benthamiana* TPL

TPL and the mechanism by which it brings about transcriptional repression is conserved across many plants (Causier et al., 2012). To determine whether the interaction between HaRxL21 and TPL is more widespread than just *A. thaliana*, interaction with TPL from 2 hosts of the related oomycete pathogen *Phytophthora infestans* (*Pi*); *Nicotiana benthamiana* (*Nb*) and potato (*Solanum tuberosum*, *St*) was tested. Testing for interaction with TPL from *Nb* is particularly useful as it may allow future insights about the mechanism by which HaRxL21 manipulates the host plant to be studied using transient protein over-expression in *Nb*.

Clones of TPL from *Nb* and the N-terminus of *St*TPL were provided by Hazel McLellan. The amino acid sequence of the 3 clones, plus TPLΔCTLH from *A. thaliana* was aligned using T-Coffee multiple sequence alignment program (Notredame et al., 2000). Figure 4.11 shows the first 150 amino acids of this alignment. It can be observed that the CTLH domain of TPL from *A. thaliana*, *Nb* and *St* is conserved with no amino acid substitutions, however immediately after this domain amino acid substitutions exist and divergence of *A. thaliana* TPL can be observed.

```
AtTPL          MSSLSRELVFLILQFLDEEKFKETVHKLEQESGFFFNMKYFEDEVHNGNW
AtTPL-CTLH     MSSLSRELVFLILQFLDEEKFKETVHKLEQESGFFF--------------
NbTPL          MSSLSRELVFLILQFLDEEKFKETVHKLEQESGFFFNMKYFEDEVHNGNW
StTPL          MSSLSRELVFLILQFLDEEKFKESVHKLEQESGFFFNMKYFEDEVHNGNW
               ************************:************

AtTPL          DEVEKYLSGFTKVDDNRYSMKIFFEIRKQKYLEALDKHDRPKAVDILVKD
AtTPL-CTLH     ----------------------------------KHDRPKAVDILVKD
NbTPL          DEVEKYLSGFTKVDDNRYSMKIFFEIRKQKYLEALDKRDRSKGVEILVKD
StTPL          DEVEKYLSGFTKVDDNRYSMKIFFEIRKQKYLEALDKRDRSKGVEILVKD
                                                *:**.*.*:*****

AtTPL          LKVFSTFNEELFKEITQLLTLENFRENEQLSKYGDTKSARAIMLVELKKL
AtTPL-CTLH     LKVFSTFNEELFKEITQLLTLENFRENEQLSKYGDTKSARAIMLVELKKL
NbTPL          LKVFASFNEELFKEITQLLTLENFRENEQLSKYGDTKSARAIMLVELKKL
StTPL          LKVFASFNEDLFKEITQLLTLDNFRENEQLSKYGDTKSARAIMLVELKKL
               ****::***:**********:******************************
```

Figure 4.11: Protein sequence alignment between *A. thaliana* TPL, *Nb*TPL and *St*TPL.
Protein sequence alignment between the first 150 amino acids of *A. thaliana* TPL, *Nb*TPL and *St*TPL using T-Coffee multiple sequence alignment program (http://www.ebi.ac.uk/Tools/msa/tcoffee/). *A. thaliana* TPLΔCTLH is included to show the location of the CTLH domain.

It was found by Y2H that HaRxL21 was able to interact with both *St*TPL and *Nb*TPL, although the interaction with *St* TPL was only observed between DB:HaRxL21 and AD:*St*TPL (figure 4.12). No autoactivation was observed for either of these TPL clones.

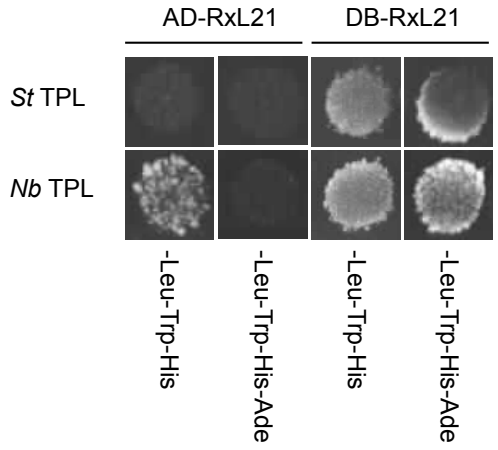


Figure 4.12: Interaction of HaRxL21 with *Nb*TPL and *St*TPL.
Interaction between HaRxL21 with *Nb*TPL and *St*TPL by Y2H. Yeast growth is shown on plates requiring activation of the His and Ade reporter genes.

4.3.4 EAR motif mutagenesis

Site-directed mutagenesis (SDM) of the EAR motif was performed to further characterise the importance of individual amino acid residues in this motif.

Residues were mutated to Phenylalanine (Phe/F). Phe was chosen because of a study in which the EAR motif in the protein SUPERMAN was characterised (Hiratsu et al., 2004). In this study, the 3 Leucine (Leu/L) residues in the EAR motif (Leu354, Leu356 and Leu358) were mutated to F individually, in pairs or all together. By mutating the amino acids in the EAR motif of HaRxL21 in the same way it was hoped that comparisons can be made to this study.

Leu residues were also mutated to Alanine (Ala/A) and Isoleucine (Ile/I). Alanine substitution was used to ensure that removal of interaction was not due to steric hindrance after amino acid substitution (Morrison and Weiss, 2001). Ile was also chosen because it is the amino acid which shares the most properties with Leucine (they are both aliphatic, hydrophobic, non aromatic and non-charged) (Livingstone and Barton, 1993). Substitution to Ile will therefore determine whether an amino acid with similar properties could facilitate the interaction of HaRxL21 and TPL.

Mutating all three Leu residues in the EAR motif of HaRxL21 (either to Phe, Ala or Ile) was found to abolish interaction between AD::TPL and DB::HaRxL21 (figure 4.13). Substituting any combination of two of the three Leu residues in was also found to be sufficient to prevent interaction. When the Leu residues were individually changed, the interaction was found to occur to different extents. The same mutated EAR motif constructs were tested for interaction with *St* and *Nb*TPL. The same pattern of interaction

was found to exist for *Nb*TPL as with TPL from *A. thaliana*. However a different pattern was observed with *St*TPL. The interaction was not abolished with any single amino acid substitutions and only 2 out of 3 combinations of double substitutions prevented the interaction from occurring.

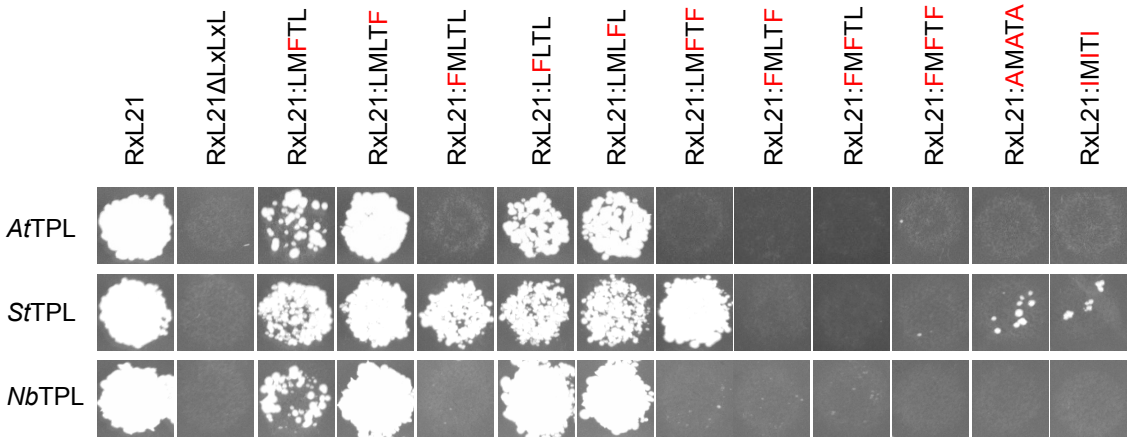


Figure 4.13: Site-directed mutagenesis of Leucine residues in the EAR domain of HaRxL21 causes reduces interaction strength or prevents interaction with TPL.
Site directed mutagenesis between pDB HaRxL21 and pAD TPL.

A dilution series was then performed to better quantify interaction strength due to the contribution of individual amino acids in the EAR motif. Mated colonies (containing AD::TPL and DB::RxL21 variants) were picked from the -Leu -Trp plate, these were then grown up for 48 h in media lacking Leu and Trp. Cultures were then adjusted to the same OD in media lacking Leu, Trp and His and a dilution series plated on -Leu -Trp plates to confirm that the same amount of yeast was present in each sample (figure 4.14).

They were then grown for 48 h in -Leu -Trp -His media and a dilution series plated after 48 h (figure 4.14). It can be observed that mutation of the Leu358 residue in the EAR motif does not appear to alter yeast growth compared to the full length effector. The M and T residues between the Leu residues also have little effect on the interaction strength.

4.3.5 EAR motif containing effectors

TPL and TPRs are recruited by proteins containing transcriptional repression domains in a wide range of plants. Because of this broad range, it is plausible that this machinery could be a common target of pathogen effector proteins. To test whether transcriptional repression via the EAR motif is a mechanism commonly used by oomycete effector proteins, the presence of the EAR motif (LxLxL) in candidate effectors from the *Hpa* genome

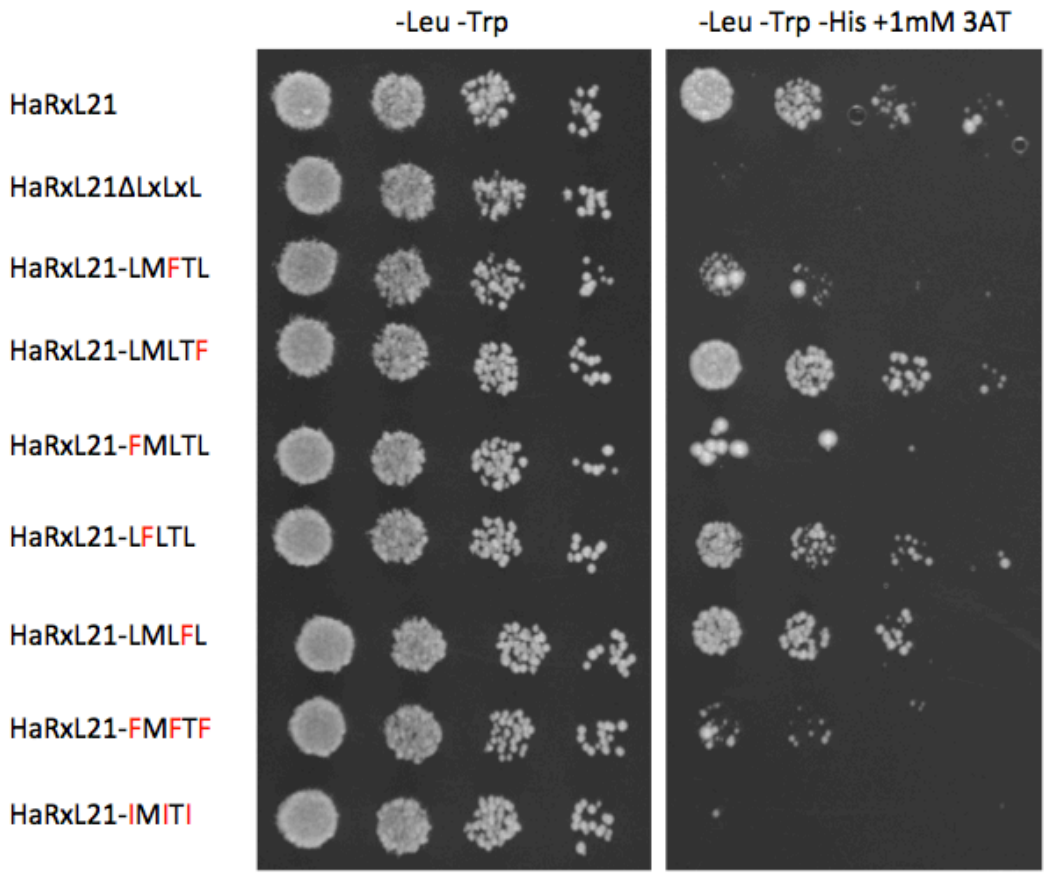


Figure 4.14: Site-directed mutagenesis of Leucine residues in the EAR domain of HaRxL21 causes reduced interaction strength with TPL.
Dilution series were performed to determine interaction strength when individual amino acid residues were altered by site-directed mutagenesis. between pDB HxRxL21 and pAD TPL. Growth on plates lacking histamine (-His) indicates activation of the reporter gene by protein-protein interaction, 3AT is added asa competitive inhibitor of the HIS3 gene product.

was investigated.

The amino acid sequence of RxLR and RxL-like (RxLL) candidate effector proteins encoded by *Hpa* were screened for the presence of the LxLxL motif (using a Perl script, Jens Steinbrenner). SignalP (Petersen et al., 2011) was then used to identify predicted signal peptide cleavage sites and any RxLs or RxLLs containing an LxLxL motif predicted to be within the signal peptide were not included in further work.

The LxLxL motif was found to be present in 16 RxLs and 35 RxLLs, of these, the sequence of 2 RxLs and 4 RxLLs were also found to contain multiple LxLxL motifs. The position of the LxLxL motif was also noted, since the EAR motif has been at the N or C terminus in examples of functional EAR motif containing proteins (Tiwari et al., 2004; Pauwels et al., 2010; Shyu et al., 2012). The LxLxL motif of 5 RxLs (including HaRxL21) and 7 RxLLs were found to be within 35 amino acids of the C-terminus of the protein. No RxLs or RxLLs were found to have the LxLxL motif at the N terminus, except those which were predicted to be within the signal peptide. In addition to the nuclear localisation of HaRxL21 (figure 4.8a), HaRxL48, HaRxL55, HaRxLL100 and HaRxLL470 have also been found to be nuclear localised in *A. thaliana*, suggesting a role in manipulating host transcription (Laura Lewis, personal communication). RxLs and RxLLs predicted to contain the EAR motif are detailed in table 4.1. The Pfam matches for these RxLs and RxLLs are also shown (Punta et al., 2012), although there is no commonly found Pfam match between this subset of effector candidates.

Table 4.1: *Hpa* RxLs and RxLLs found to contain the LxLxL motif.

Name	Type	Location	Multiple LxLxL	Pfam Matches	Nuclear	Cytoplasmic
HaRxL021	RxL	C			x	
HaRxL048	RxL	C			x	
HaRxL053	RxL	C		Ndr		
HaRxL083	RxL	C	Yes			
HaRxL111	RxL	C	Yes			x
HaRxL001	RxL	Mid				
HaRxL011	RxL	Mid				
HaRxL019	RxL	Mid		Prefoldin_2		
HaRxL024	RxL	Mid				
HaRxL035	RxL	Mid		DUF2435, DUF2411, Spheroidin		
HaRxL055	RxL	Mid			x	
HaRxL095	RxL	Mid				
HaRxL109	RxL	Mid				x
HaRxL116	RxL	Mid		UPF0128		
HaRxL117	RxL	Mid				

Name	Type	Location	Multiple LxLxL	Pfam Matches	Nuclear	Cytoplasmic
HaRxL136	RxL	Mid				
HaRxLL006	RxLL	C				
HaRxLL029	RxLL	C				
HaRxLL039	RxLL	C				
HaRxLL076	RxLL	C				
HaRxLL100	RxLL	C		DUF2390	x	
HaRxLL165	RxLL	C				
HaRxLL483	RxLL	C		Pkinase		
HaRxLL001	RxLL	Mid	Yes	Peptidase_S8, DUF1191		
HaRxLL014	RxLL	Mid				
HaRxLL019	RxLL	Mid				
HaRxLL020	RxLL	Mid		Nrf1_DNA-bind		
HaRxLL022	RxLL	Mid				
HaRxLL025	RxLL	Mid				
HaRxLL031	RxLL	Mid				
HaRxLL032	RxLL	Mid				
HaRxLL034	RxLL	Mid				
HaRxLL036	RxLL	Mid	Yes			
HaRxLL046	RxLL	Mid				
HaRxLL049	RxLL	Mid				
HaRxLL061	RxLL	Mid				
HaRxLL073	RxLL	Mid		CcdA		
HaRxLL093	RxLL	Mid		RVT_2		
HaRxLL101	RxLL	Mid				
HaRxLL120	RxLL	Mid	Yes			
HaRxLL131	RxLL	Mid		Abhydrolase_1, FSH1, Armet		
HaRxLL147	RxLL	Mid				
HaRxLL151	RxLL	Mid				
HaRxLL164	RxLL	Mid				
HaRxLL172	RxLL	Mid				
HaRxLL181	RxLL	Mid				
HaRxLL452	RxLL	Mid				
HaRxLL465	RxLL	Mid				
HaRxLL470	RxLL	Mid		BTAD, TPR_2	x	
HaRxLL489	RxLL	Mid				
HaRxLL490	RxLL	Mid	Yes	UCH, zf-ranBP, DUF2464,		

Table showing LxLxL-containing RxL and RxLL effectors. LxLxL motifs are categorised as being located within 35 amino acids of the 'N' or 'C'-terminus or otherwise 'Mid'. Where shown,

effectors were localised in *A. thaliana* by Laura Lewis.

A subset of RxL and RxLL effectors were previously cloned into pAD and pDB by Rachel Clewes and Rebecca Allen. The interaction of these proteins with TPL was tested by Y2H in both directions. No EAR motif containing effectors other than HaRxL21 tested were found to interact with TPL by Y2H (figure 4.15). As a positive control, the interaction of TPL with HaRxL21 and the dimerisation ability of TPL was also confirmed.

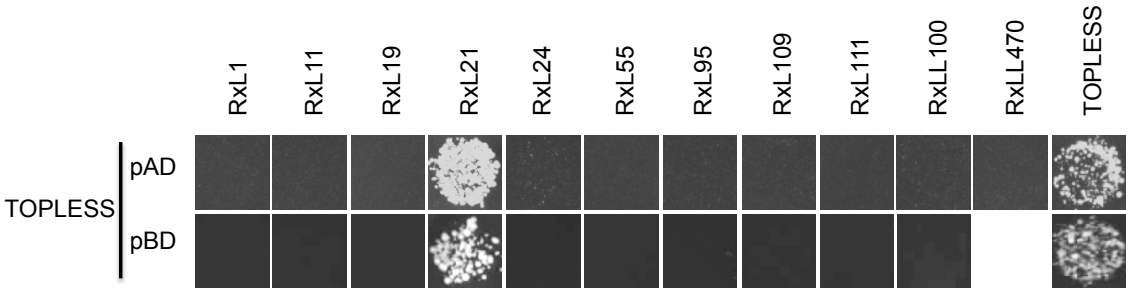


Figure 4.15: HaRxL21 is the only EAR motif containing RxL or RxLL tested to interact with TPL.
Y2H was performed between EAR motif-containing RxL and RxLLs and TPL. Of these, HaRxL21, HaRxL55, HaRxLL100 and HaRxLL470 are nuclear localised in *A. thaliana*. Yeast growth is shown on -Leu -Trp -His + 1mM 3AT media. In all cases, yeast growth was observed on -Leu -Trp media, indicating successful mating.

4.4 *in planta* validation of effector targets

4.4.1 Split YFP

One system which can be used for *in planta* validation of protein-protein interactions is that of Bimolecular fluorescence complementation (BiFC). Here, split-YFP was used, the premise behind this is that either half of the yellow fluorescent protein (YFP) are fused to each of the proteins interacting partners to be verified. Interaction of these proteins will then bring the N and C-terminal halves of E-YFP into close proximity, resulting in fluorescence. The Gateway compatible pBiFP (BiFC *in Planta*) vectors used here were kindly provided by Franois Parcy (University Grenoble, France). It is known that the interaction of HaRxL21 with TPL is mediated by the EAR motif at the C-terminus of the effector, therefore HaRxL21 was cloned into a vector with an N-terminally fused YFP fragment. Here, both pBIFP2 and pBIFP3 produce N-terminal fusions to the protein of interest; pBIFP2 fuses N-YFP (the N-terminal fragment of E-YFP) and pBIFP3 fuses C-YFP (the C-terminal fragment of E-YFP).

A. tumefaciens expressing N and C fragments of E-YFP fused to the interacting proteins to be verified were infiltrated into *Nicotiana benthamiana* (method described in section 2.2.13). These were co-infiltrated with p19 silencing suppressor (described by Voinnet et al. (2003)) to prevent post-transcriptional gene silencing of the expressed constructs. Leaves were then imaged as described in section 2.2.12. Interaction of TCP14 was not tested using BIFC due to the inability to confirm interaction with HaRxL21 using Y2H.

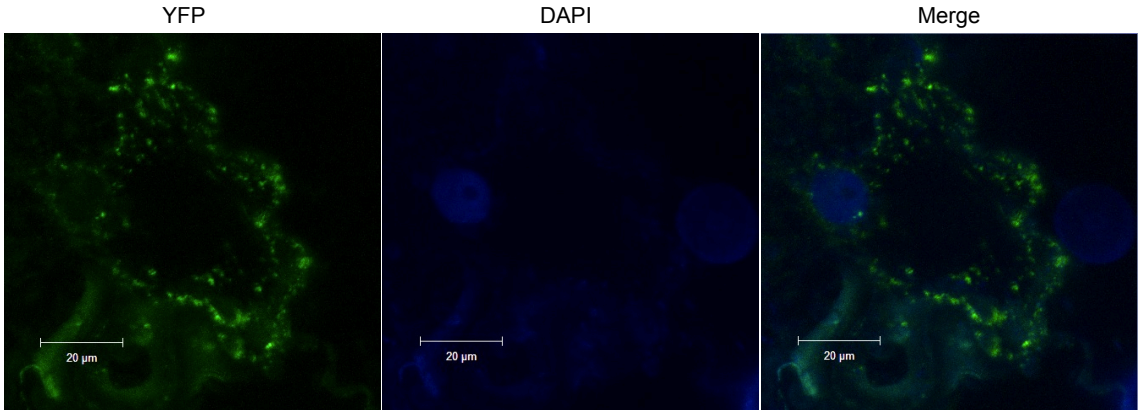
4.4.1.1 SWAP

The interaction of HaRxL21 with SWAP was also tested using BIFC. This interaction was tested in two orientations; BIFC3::HaRxL21 and BIFP2::SWAP, and BIFP2::HaRxL21 and BIFC3::SWAP. No fluorescence was observed in either case, indicating that *in planta* validation of the interaction of HaRxL21 with SWAP was not possible using BIFC.

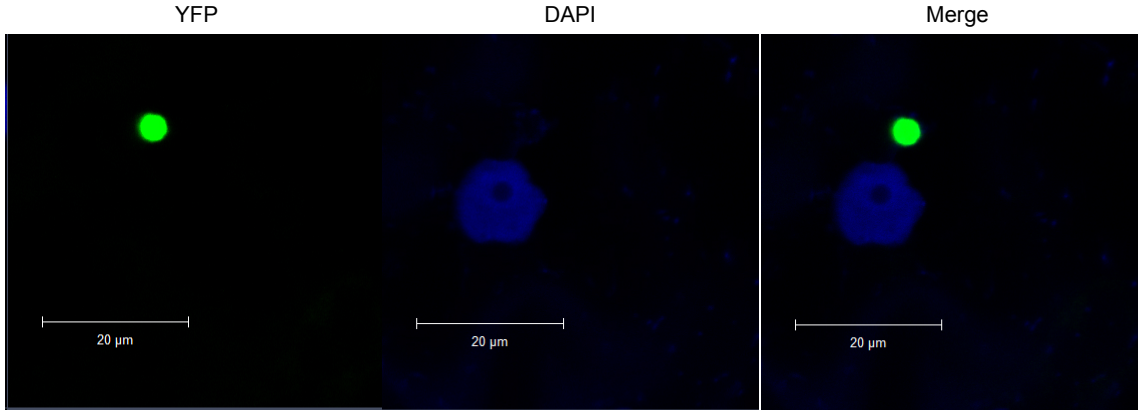
4.4.1.2 OBE1

The interaction of HaRxL21 with OBE1 was also tested using split YFP. This interaction was tested in both orientations; BIFC3::HaRxL21 and BIFP2::OBE1 (figure 4.16a) and BIFP2::HaRxL21 and BIFC3::OBE1 (figure 4.16b). When HaRxL21 was N-terminally fused to the C half of E-YFP, fluorescence was observed. However the localisation did

not occur in the nucleus as might be expected by the nuclear localisation of both OBE1 and HaRxL21.



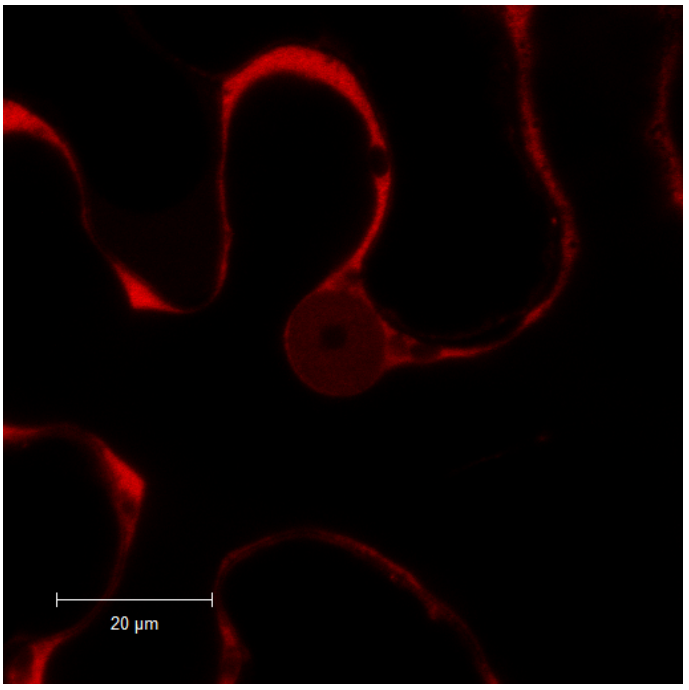
(a)



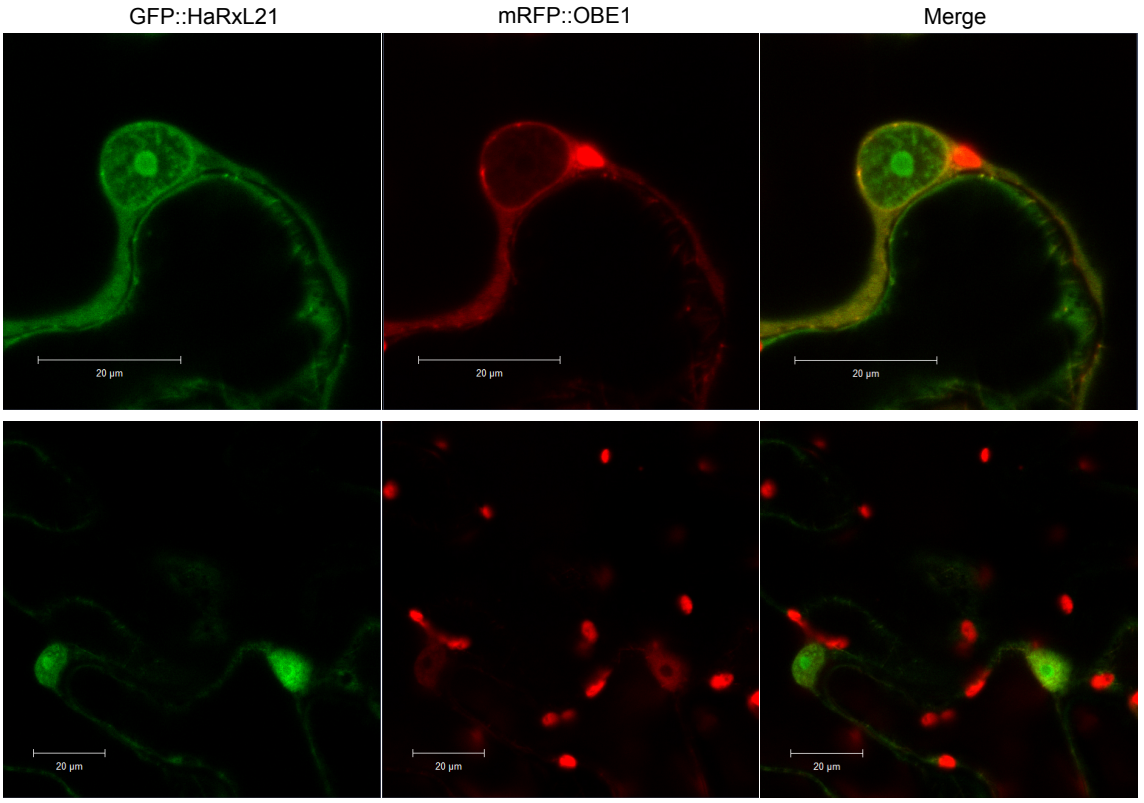
(b)

Figure 4.16: HaRxL21 interacts with OBE1 using split YFP, but not in the nucleus. BIFC was performed using *Agrobacterium tumefaciens* mediated transient expression of BIFP constructs in *Nicotiana benthamiana*; (a) BIFC3::HaRxL21 and BIFP2::OBE1 (b) BIFP2::HaRxL21 and BIFC3::OBE1. Nuclear localisation is shown by DAPI.

Images taken by Jens Steinbrenner confirmed that, as observed by Saiga et al. (2008), OBE1 localises to the nucleus and the cell membrane (figure 4.17a). When co-infiltrated with GFP::HaRxL21, mRFP::OBE1 localises to the nucleus and the membrane, but in addition can be observed to show localisation external to the nucleus, such as in the chloroplasts. It can be observed that GFP::HaRxL21 does not re-localise to show co-localisation with mRFP::OBE1 external to the nucleus and in some cases is excluded from the areas where mRFP::OBE1 is accumulating (figure 4.17b).



(a)



(b)

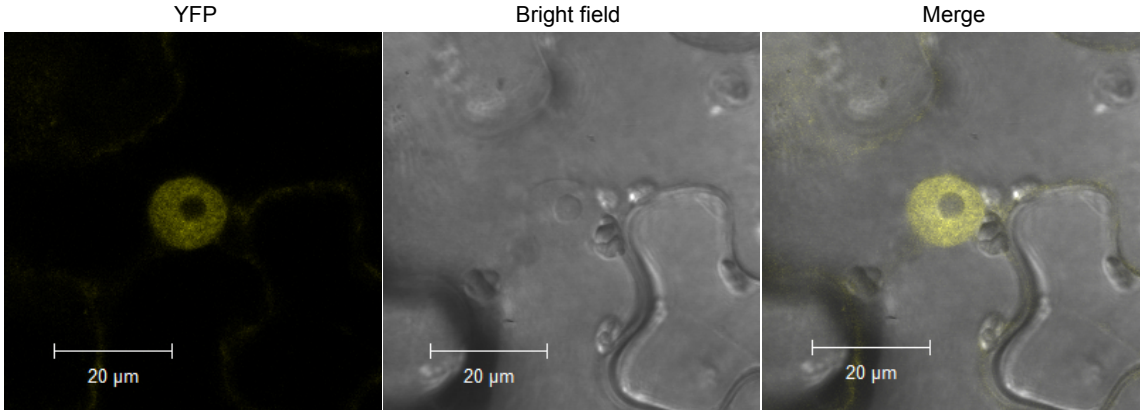
Figure 4.17: Localisation of OBE1 alone and in the presence of HaRxL21.
OBE1 localisation in *Nicotiana benthamiana*; (a) mRFP::OBE1 (b) Co-infiltration of mRFP::OBE1 (red) and GFP::HaRxL21 (green). Images taken by Jens Steinbrenner.

4.4.1.3 TPL

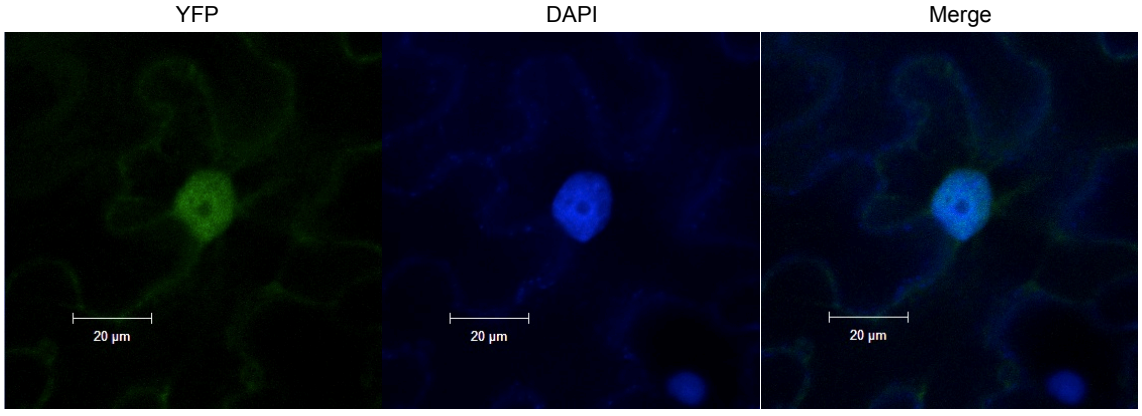
The interaction between BIFP2::HxRxL21 and BIFP3::TPL was tested and E-YFP fluorescence observed in the nucleus, but not in the nucleolus. This was visualised by co-localisation with the DNA stain DAPI (Kapuscinski, 1995), in addition to visualisation using bright field microscopy (figure 4.18a and b). When imaging of DAPI and E-YFP, separate lasers were used to ensure no crosstalk when imaging. No fluorescence was observed when BIFP2::HaRxL21ΔLxLxL and BIFP3::TPL were expressed, indicating no interaction was occurring (Figure 4.18c) as no change in stability of HaRxL21ΔLxLxL has previously been shown (Figure 4.8b). The interaction between HaRxL21 and TPL was therefore verified *in planta* by this method.

It was noted that the EAR motif was conserved between HaRxL21 alleles from the *Hpa* isolates Emoy2 and Cala2 (figure 4.9). However differences in the protein structure between these two alleles were observed due to the presence of amino acid substitutions in the protein sequence (figure 4.10), and HaRxL21 Cala was not found to interact with TPL using Y2H. To verify whether these differences between the two alleles were observed only in yeast or replicated *in planta*, the interaction of HaRxL21 Cala and TPL was therefore tested by BIFC. There was found to be an interaction between HaRxL21 Cala and TPL which occurred in the nucleus, shown by the presence of E-YFP fluorescence which co-localised with DAPI stain (figure 4.19a).

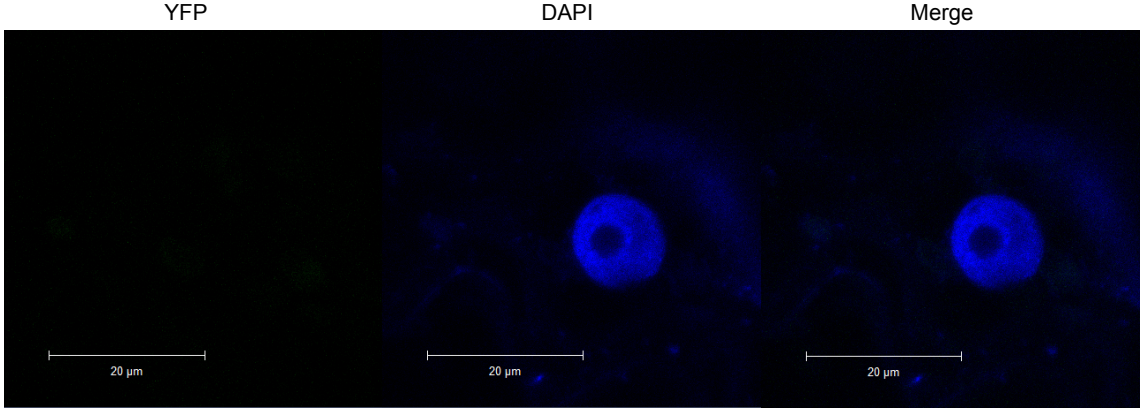
Interaction of HaRxL21 with TPL from *Nicotiana benthamiana* was also tested using BIFC. Interaction between BIFP2::HxRxL21 and BIFP3::*Nb*TPL was found to cause E-YFP fluorescence in the nucleus (figure 4.19b).



(a)



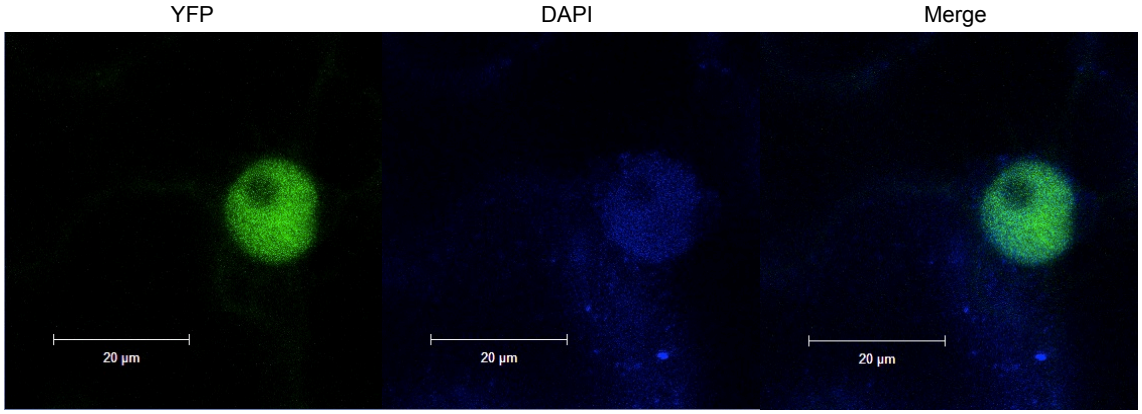
(b)



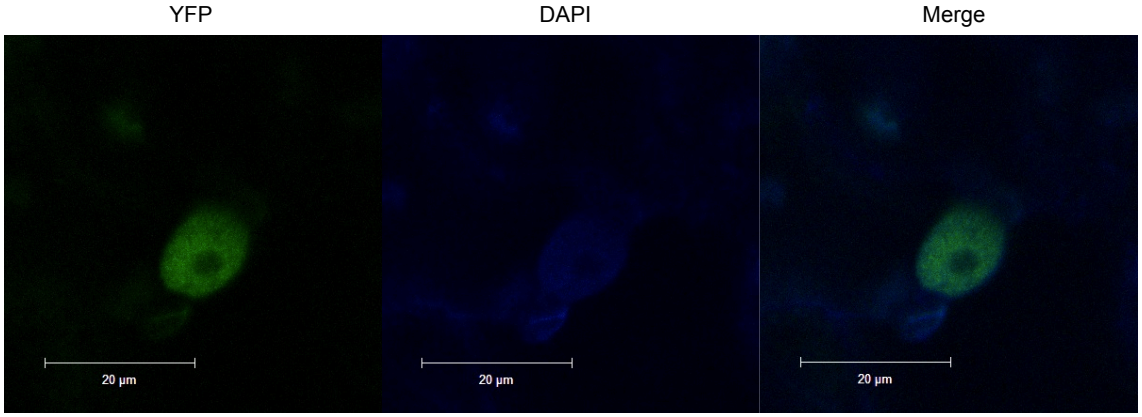
(c)

Figure 4.18: HaRxL21 (but not HaRxL21ΔLxLxL) interacts with TPL in the nucleus using split YFP.

BIFC was performed using *Agrobacterium tumefaciens* mediated transient expression using BiFC *in Planta* vectors in *Nicotiana benthamiana*; (a,b) BIFP2::HxRxL21 and BIFP3::TPL. Nuclear localisation is shown by (a) bright field imaging and (b) DAPI. (c) BIFP2::HxRxL21ΔLxLxL and BIFP3::TPL shows no E-YFP fluorescence.



(a)



(b)

Figure 4.19: HaRxL21 Cala allele interacts with TPL and HaRxL21 interacts with TPL from *Nicotiana benthamiana* in the nucleus using BIFC.

BIFC was performed using *Agrobacterium tumefaciens* mediated transient expression of BIFP constructs in *Nicotiana benthamiana*; (a) BIFP2::HxRxL21 Cala and BIFP3::TPL (b) BIFP2::HxRxL21 and BIFP3::*Nb*TPL. Co-localisation with DAPI is shown.

4.4.2 Co-Immunoprecipitation

Co-immunoprecipitation (Co-IP) was performed to further validate protein-protein interactions *in planta*. Because TPL and HaRxL21 have been shown to interact in the nucleus rather than diffusely throughout the cell (Figure 4.18), nuclear enrichment was performed to produce a strong enough signal for detection through Co-IP (as described in section 2.2.11.3 and section 2.2.11.3). HA::TPL and C-YFP::HaRxL21 were transiently over-expressed in *N. benthamiana*. C-YFP::HaRxL21 uses the BIFC vector BIFP3 (section 2.1.6) and can be used as it is recognised by the α-GFP-HRP antibody used here (Jens Steinbrenner, Methods in Enzymology, 2014 in press).

HA::TPL was immunoprecipitated by α-HA beads and C-YFP::HaRxL21 was detected by western blot using α-GFP when co-expressed with HA::TPL, but not when co-expressed with HA::TPLΔCTLH (Figure 4.20). It was observed that detection of TPLΔCTLH or full length TPL in the absence of full length HaRxL21 was lower than when full length TPL and HaRxL were co-expressed (Figure 4.20).

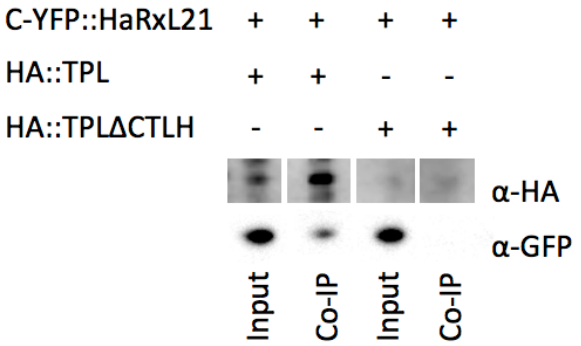


Figure 4.20: HA::TPL pulls down HaRxL21.
HA::TPL and C-YFP::HaRxL21 interact *in planta*. Nuclear protein was extracted from *N. benthamiana* transiently expressing HA::TPL and C-YFP::HaRxL21, HA::TPL was immunoprecipitated using α-HA beads. Total protein (Input) and eluate from α-HA beads (Co-IP) was analysed by western blot using α-HA and an α-GFP antibody which recognises the C-terminus of YFP.

4.5 Pathology implications of protein-protein interactions

4.5.1 *Hpa* phenotype

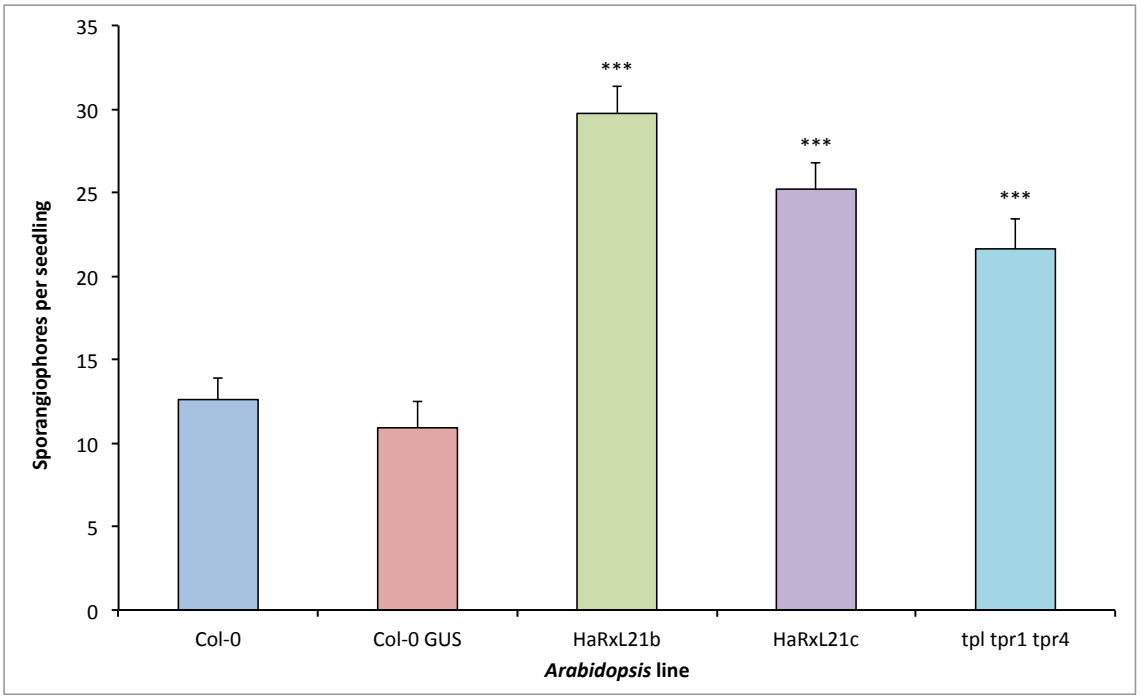
To assess the importance of protein-protein interaction targets, it was investigated whether there was any susceptibility change conferred to *Hyaloperonospora arabidopsidis* (*Hpa*) by knocking out confirmed interaction targets of HaRxL21.

Zhu et al. (2010) have shown that TPL, TPR1 and TPR4 function redundantly in plant defense, and that Col-0 *tpr1-tpl-tp4 A. thaliana* plants show enhanced susceptibility to *P. syringae*. *A. thaliana* line Col-0 *tpr1-tpl-tpr4* was kindly provided by Zhu et al. (2010). Fourteen day old seedlings of Col-0 *tpr1-tpl-tp4*, in addition to Col-0 and Col-0 GUS controls were sprayed with *Hpa* isolate Noks1 and sporangiophores were counted 4 days post infection (method described in section 2.2.6.3). HaRxL21b and c plants were also included as a positive control since these lines have been found to enhance susceptibility to *Hpa*. Knocking out TPL, TPR1 and TPR4 was found to enhance susceptibility to *Hpa* compared to wild type and Col-0 GUS controls (figure 4.21a).

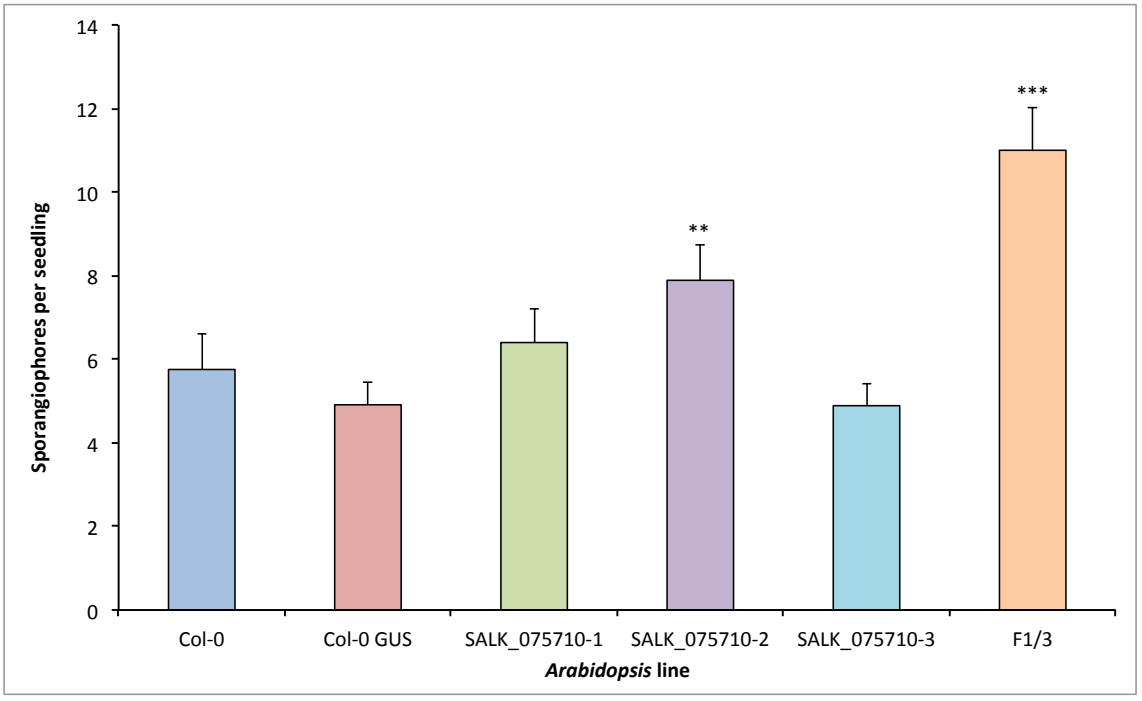
Three homozygous OBE1 T-DNA insertion lines (derived from SALK_075710 and genotyped by Tine Payne) were tested for susceptibility to *Hpa*. The line F1/3 (35S::HaRxL14) is included as a positive control as it is known to have enhanced susceptibility to *Hpa* (Jens Steinbrenner, personal communication). One of the three lines (SALK_075710-2) was found to support significantly more sporangiophores per seedling than Col-0 GUS, but the difference to Col-0 was not significant. SALK_075710-1 and SALK_075710-3 did not show any significant difference in susceptibility to Col-0 or Col-0 GUS. The F1/3 positive control showed significantly more sporangiophores per seedling than Col-0 and Col-0 GUS (figure 4.21b).

4.5.2 *B. cinerea* phenotype

To determine whether the increased susceptibility to *B. cinerea* observed on HaRxL21 expressing plants could be explained by interaction with TPL, *B. cinerea* pathogenicity on Col-0 *tpr1-tpl-tpr4* plants was examined using the method described in section 2.2.10.2. It was found that Col-0 *tpr1-tpl-tpr4* plants showed a significantly higher lesion area than Col-0 at 48, 72 and 96 h post infection (Figure 4.22a). It was also observed that lesions on *tpr1-tpl-tpr4* plants were more advanced and showing more sporulation (Figure 4.22b).



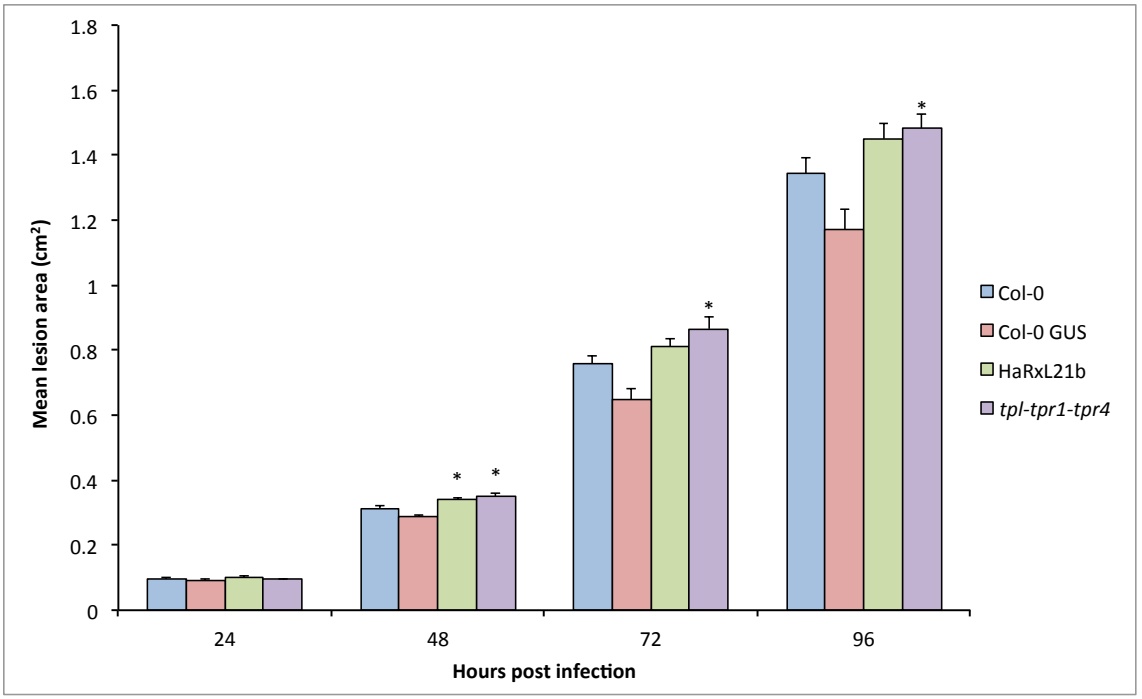
(a)



(b)

Figure 4.21: The *Hpa* phenotype of *A. thaliana* knockout lines for *tpr1-tpl-tpr4* and *OBE1*.

Hpa phenotype of (a) Col-0 *tpr1tpltpr4* (b) three homozygous insertion lines derived from SALK_075710 compared to Col-0 and Col-0 GUS controls. Positive controls for enhanced susceptibility (a) HaRxL21b and HaRxL21c and (b) F1/3 are also shown. Significant differences to Col-0 GUS are marked; *p<0.05; **p<0.01; ***p<0.001, error bars display standard error, n=45.



(a)

Col-0 Col-0 GUS *tpr1-tpl-tpr4*

(b)

Figure 4.22: TPL knockouts show an increased susceptibility to *B. cinerea*

B. cinerea susceptibility phenotype of Col-0 *tpr1-tpl-tpr4* plants compared to Col-0 and Col-0 GUS. (a) Lesion size and (b) Lesion appearance 96 h post infection. (Significant differences to Col-0 using a T-test are shown; **p*<0.05; ***p*<0.01, error bars display standard error, n=24).

4.5.3 TPL EDV phenotype

Previously (Figure 3.13), it has been shown that HaRxL21 causes enhanced susceptibility compared to AvrRPS4-AAAA control (as used in Fabro et al. (2011)) when delivered via the effector detector vector (EDV) system for delivering effectors via the type III secretion system of *Pseudomonas syringae* (Sohn et al., 2007) (method described in section 2.2.8.2).

The same EDV constructs were used here, but were infiltrated into Col-0 *tpr1-tpl-tpr4* plants (Zhu et al., 2010), rather than Col-0. It was observed that there was no difference in susceptibility between delivery of HaRxL21 compared to the AvrRPS4-AAAA control (figure 4.23). This result contrasts the previous susceptibility advantage on Col-0 plants due to HaRxL21 expression.

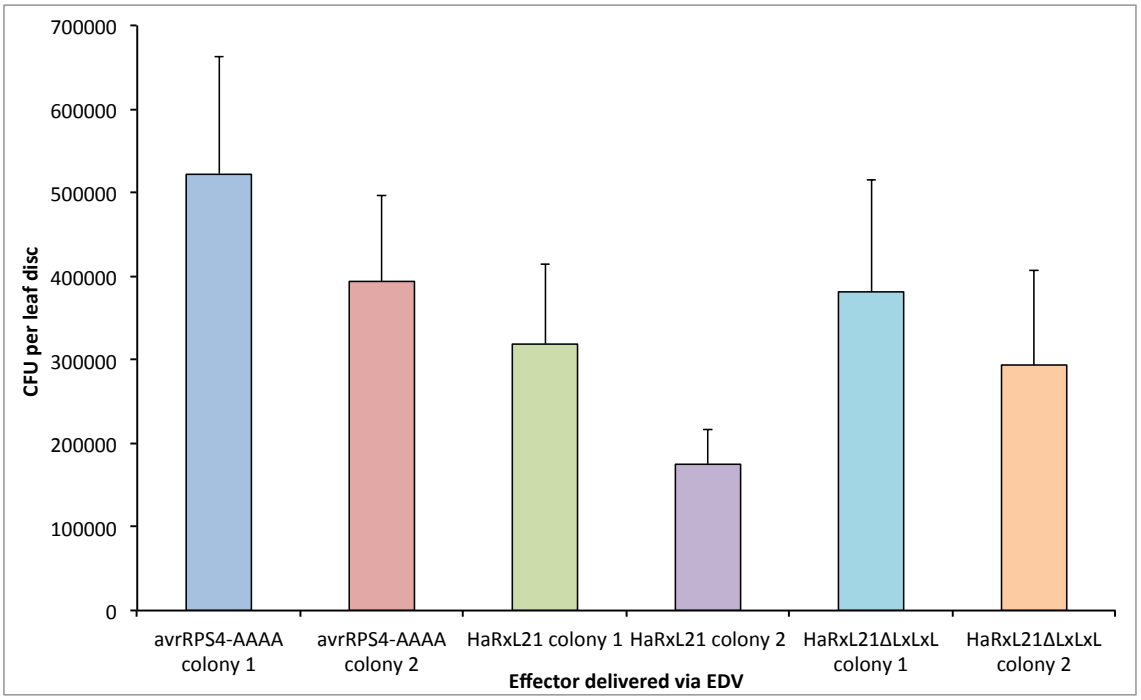


Figure 4.23: EDV::HaRxL21 does not have a susceptibility advantage on *tpr1-tpl-tpr4 A. thaliana* plants.
Using EDV, avrRPS4-AAAA, HaRxL21 and HaRxL21ΔLxLxL were delivered via the *P. syringae* type III secretion system into *tpr1-tpl-tpr4* plants. Day 0 colony counts were equal across all treatments. Colony counts are shown 72 h post infection, error bars display standard error (n=4), no significant differences were found using a T-test.

4.5.4 Comparison between HA::RxL21 and HA::HaRxL21ΔLxLxL plants

Deletion of the EAR motif prevents interaction of HaRxL21 with TPL (Figure 4.6). The aim here was therefore to establish whether the EAR motif (and therefore interaction with TPL) is required for the previously described susceptibility advantage conferred to *Hpa* by *in planta* expression of HaRxL21 (Figure 3.5a).

HaRxL21 and HaRxL21ΔEAR were cloned into pEG201; a Gateway-compatible vector for *in planta* expression with an N-terminal HA tag (Earley et al., 2006). A GFP control were also cloned into this vector. Constructs were transformed into *A. thaliana* ecotype Col-4 by floral dipping as described in section 2.2.2. Three independently transformed homozygous lines were chosen for further characterisation. HA::HaRxL21-4,-6 and -9 and HA::HaRxL21ΔEAR-1,-8 and -9 denote homozygous plant lines generated from independent transformations.

4.5.5 Protein Expression Levels

To determine whether these plants were expressing HaRxL21, samples were taken from 14 day old seedlings and western blots performed with α-HA to determine protein expression levels. Expression of HA::GFP and each of the three homozygous plant lines for HA::HaRxL21 and HA::HaRxL21ΔEAR is shown in Figure 4.24. HA::GFP can be detected at a higher level than any of the HaRxL21 plants (blue arrow, Figure 4.24). To ensure equal loading, the membrane was stained with Coomasssie brilliant blue. Results show that HA::HaRxL21-4 and -9 are expressing HaRxL21 and HA::HaRxL21-6 is expressing at a negligible level. Of the HA::HaRxL21ΔEAR lines, -1 is expressing the most, -8 is expressing at a level which is comparable to HA::HaRxL21-4 and -9, and HA::HaRxL21ΔEAR-9 is showing negligible or no expression.

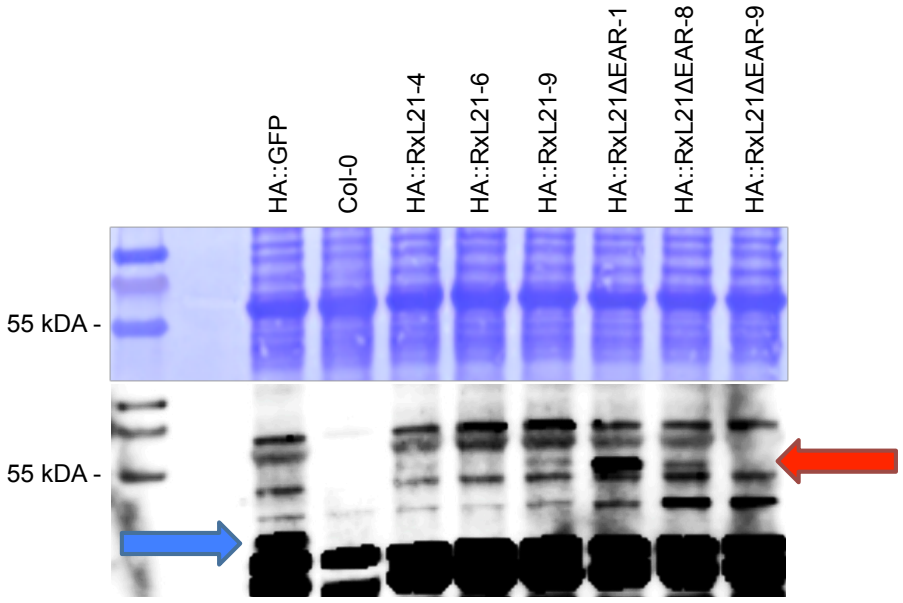


Figure 4.24: Expression of HA::HaRxL21 and HA::HaRxL21ΔEAR in *A. thaliana* plants.
(Top panel) Membrane stain with coomassie brilliant blue to show equal loading of protein samples. (Lower panel) Western blot showing expression of HA::HaRxL21 and HA::HaRxL21ΔEAR in *A. thaliana* plants. GFP is indicated by blue and the HaRxL21 band is indicated by the red arrow.

4.5.6 RNA Expression Levels

In addition to protein level in these plants, expression of *HaRxL21* was examined by quantitative reverse transcription PCR (qRT-PCR) performed by Jens Steinbrenner. Expression of *HaRxL21* was normalised to *AtAct2* and *UBQ5* for each sample. *HaRxL21* expression was was detected in all plant lines but not in Col-0 or HA::GFP (Figure 4.25). Correlation was also observed between high *HaRxL21* expression and high protein detection in HA::HaRxL21ΔEAR-1 (Figure 4.25 and Figure 4.24). It was also observed that *HaRxL21* expression was higher than in 35S::HaRxL21 lines; HaRxL21a-c.

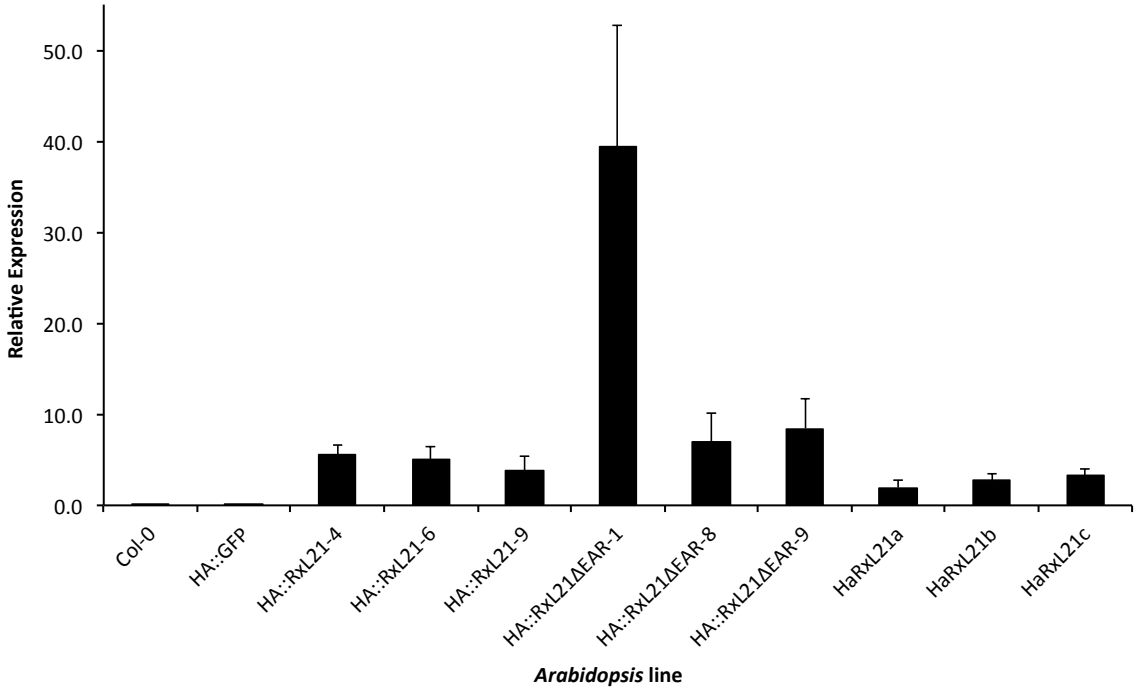


Figure 4.25: Expression levels of *HaRxL21* in HA::HaRxL21 and HA::HaRxL21ΔEAR *A. thaliana* plants.
Expression levels of *HaRxL21* (normalised to *AtAct2* and *UBQ5*) detected by quantitative RT-PCR in HA::HaRxL21 and HA::HaRxL21ΔEAR *A. thaliana* plants compared to Col-0, HA::GFP and three 35S::HaRxL21 lines; HaRxL21a-c. qRT-PCR performed by Jens Steinbrenner, error bars display standard error, n=3

4.5.7 *Hpa* Phenotype

To determine the effect of deleting the EAR motif of HaRxL21, the *Hpa* phenotype of HA::RxL21 and HA::RxL21ΔEAR was compared to controls; Col-0, Col-4 HA::GFP and F1/3 (HaRxL14 positive control for enhanced susceptibility). Fourteen day old seedlings were infected with the *Hpa* isolate Noks1 (as described in section 2.2.6.3) and sporangiophores counted four days post infection.

No significant difference in number of sporangiophores per seedling was observed between HA::RxL21-expressing plants and controls in two independent experiments (Figure 4.26a and Figure 4.26b).

No significant difference was observed between HA::RxL21ΔEAR-expressing plants with controls in one experiment (Figure 4.26c) and when repeated, all three HA::RxL21ΔEAR-expressing lines showed significantly less sporangiophores per seedling compared to Col-0 (Figure 4.26d). However it can be observed that line F1/3 (usually used as a positive control for enhanced susceptibility) is not showing enhanced susceptibility to *Hpa* in Figure 4.26b, Figure 4.26c and Figure 4.26d.

It was also noted that when growing these seedlings for infection, a lower germination rate was observed for HA::HaRxL21-4 and -6, these plants also exhibited pointy cotyledons. No phenotypic differences or reduced germination was observed between HA::HaRxL21ΔEAR and Col-0 or HA::GFP plants.

Hpa susceptibility of HA::RxL21-expressing plants was then directly compared to that of HA::RxL21ΔEAR-expressing plants. All of the HA::RxL21-expressing lines were found to be significantly more susceptible (measured by sporangiophores per seedling) compared to HA::RxL21ΔEAR-1 and -8 (Figure 4.27). In addition, HA::RxL21-4 and HA::RxL21-6 were significantly more susceptible to *Hpa* than HA::RxL21ΔEAR-9 (Figure 4.27). HA::RxL21ΔEAR-1 showed significantly less sporangiophores per seedling compared to HA::RxL21ΔEAR-8 and -9.

However no controls were included because only six plant lines can be sown per tray to be infected with *Hpa* in order to allow for sufficient replication. This means that in this experiment, conclusions cannot be drawn about sporangiophore counts relative to Col-0 and HA::GFP.

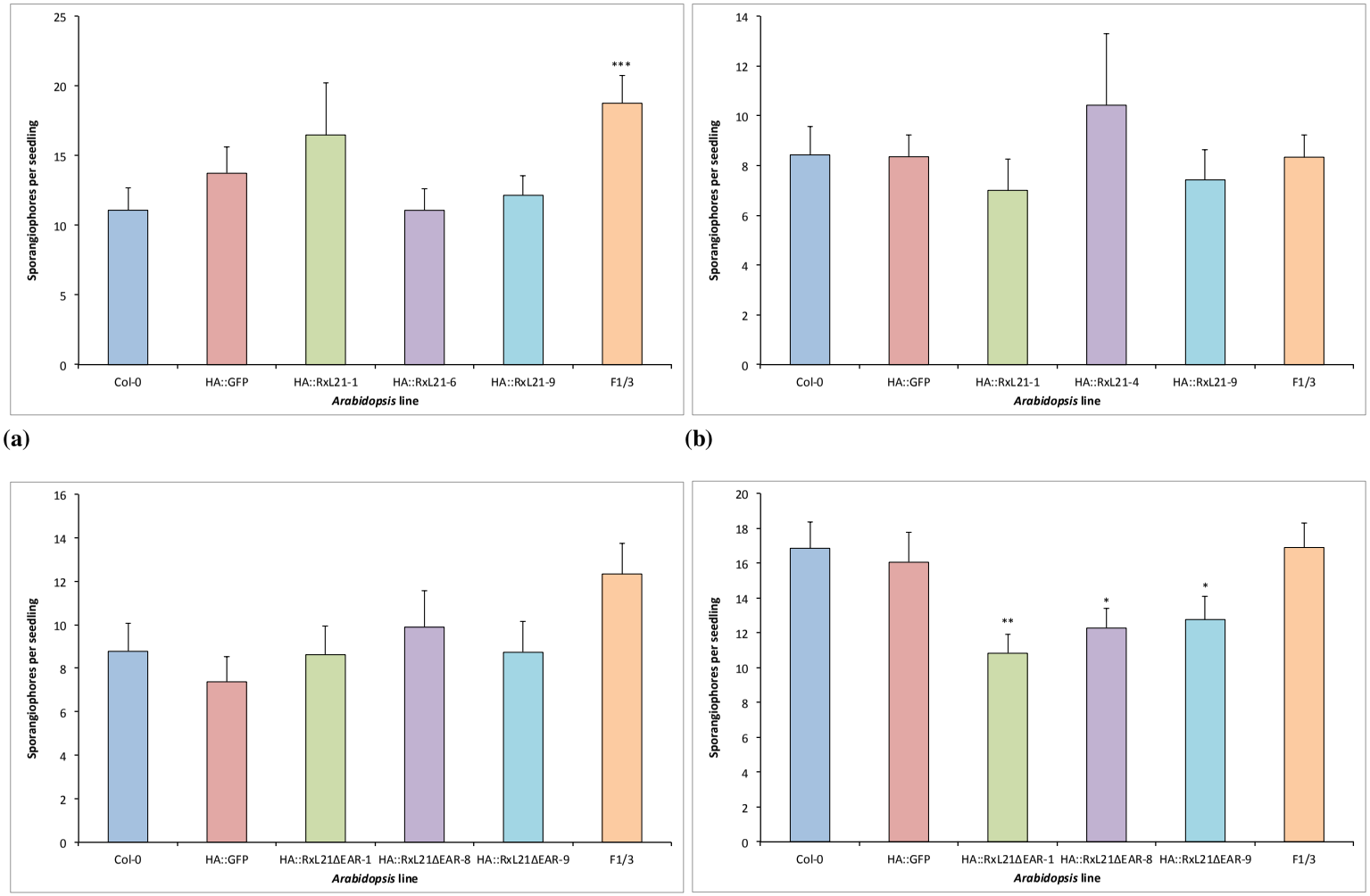


Figure 4.26: HA::HaRxL21 and HA::HaRxL21ΔEAR susceptibility to *Hpa*

Hpa spores of isolate Noks1 were sprayed on 2 week old *A. thaliana* seedlings expressing (a), (b) HA::HaRxL21 and (c), (d) HA::HaRxL21ΔEAR. Sporangiophores were counted 4 days post infection and compared to controls; Col-0 and HA::GFP, in addition to F1/3 as a positive control for enhanced susceptibility. Significant differences to Col-0 are shown; *p<0.05; **p<0.01; ***p<0.001. Error bars display standard error, n=45.

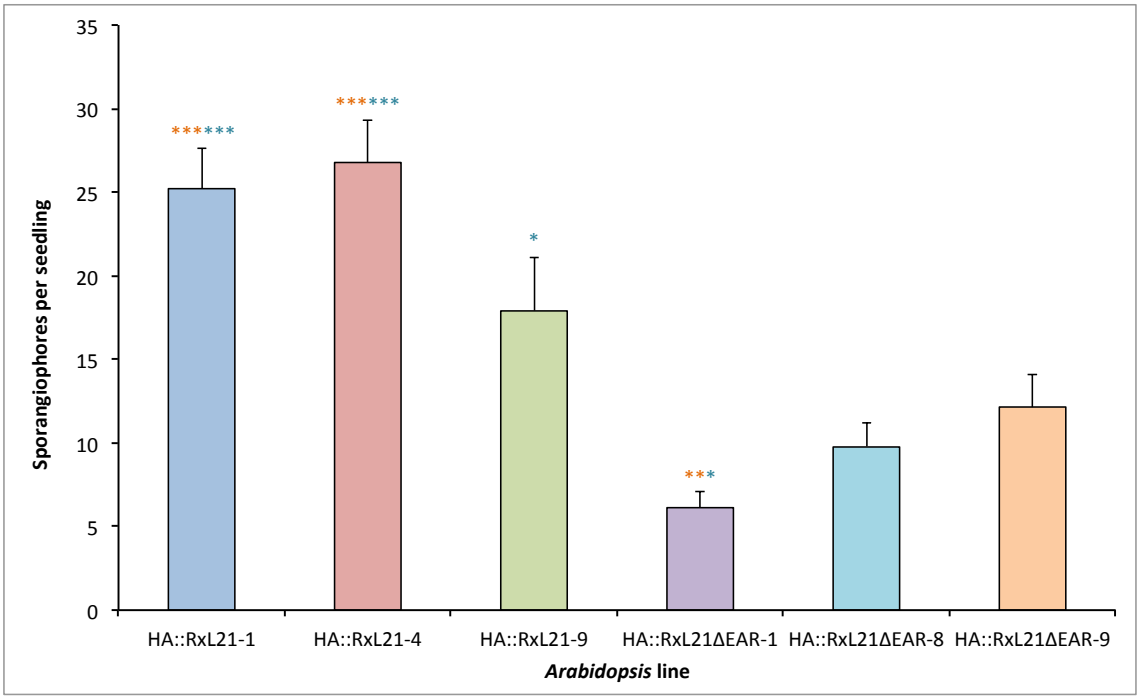


Figure 4.27: HA::RxL21 plants are more susceptible than HA::RxL21ΔEAR.
Hpa spores of isolate Noks1 were sprayed on 2 week old *A. thaliana* seedlings expressing HA::HaRxL21 and HA::HaRxL21ΔEAR and sporangiophores counted four days post infection. Significant differences to (orange) HA::RxL21ΔEAR-9 and (blue) HA::RxL21ΔEAR-8 are shown; *p<0.05; **p<0.01; ***p<0.001. All HA::HaRxL21-expressing lines had significantly more sporangiophores per seedling than HA::RxL21ΔEAR-1. Error bars show standard error, n=45.

4.6 Discussion

4.6.1 TCP14

Because TCP14 was identified to have so many protein-interaction targets involved in plant immunity, it would be a logical target for a pathogen effector protein, enabling disruption of the regulation of many defense signalling pathways. However TCP14 was found by Y2H to not interact with HaRxL21, and during a repeat of the screen it was found to weakly auto activate in the pAD vector. The ability of yeast expressing TCP14 to grow in the absence of the GAL4 DNA binding domain suggests that TCP14 may have DNA binding activity, not unexpected for a transcription factor.

TCP14 was found to be a 'hub' of the plant-pathogen interactome presented by Mukhtar et al. (2011), who used methodology by Dreze et al. (2010) for producing high quality Y2H screening, using rigorous testing and re-testing of interacting candidates. However in a large screen such as this there may be false positives. False positives in Y2H may occur due to autoactivation, or due to the interaction of proteins in yeast which would not come into contact with each other *in planta* (Vidalain et al., 2004). A final type of false-positive is the result of so-called 'sticky' proteins, the properties of which make them more likely to bind to multiple partners (Vidalain et al., 2004). These proteins are hard to distinguish from proteins with large numbers of bona fide interaction partners, and therefore may have not been accounted for in this screen. It is possible that 'hubs' such as TCP14 in the plant-pathogen interactome may therefore be results of these 'sticky' proteins. One approach which could be used to distinguish genuine interactions *in planta* would be to use an *A. thaliana* line expressing tagged TCP14 and perform pull downs to see which proteins are binding.

4.6.2 SWAP

Auto-activation is a common artefact of the Y2H system. In the case of DB::SWAP, there was growth when mated with the empty pAD vector. This means that DB::SWAP was able to activate transcription of the reporter gene without the presence of the GAL4 activation domain. Dreze et al. (2010) describe auto activators which can be a) transcription factors which contain an activation domain or b) proteins which are not transcription factors in their natural context (in this case *in planta*), however contain an activation domain and can behave as auto-activators. My data suggest that SWAP is b) as there is no previous evidence to show it is a transcription factor, despite considerable interest in transcription

factors from *A. thaliana*.

Although interaction of SWAP and HaRxL21 was not validated *in planta* by BIFC, they were repeatedly found to interact by Y2H. This interaction was observed to occur regardless of truncation of HaRxL21 to remove either the RxLR-DEER motif at the N-terminus, or to remove the EAR motif at the C-terminus. To test where this interaction occurs, smaller HaRxL21 fragments could be cloned and tested for interaction with SWAP using Y2H and BIFC. It is possible however that smaller fragments of HaRxL21 would not interact if the interaction was dependent on tertiary protein structure.

4.6.3 OBE1

The interaction of HaRxL21 and OBE1 was confirmed by Y2H and by the presence of fluorescence during BiFC. However using BIFC, this interaction was not found to occur in the nucleus. Instead, when BIFC3::HaRxL21 and BIFP2::OBE1 were co-infiltrated into *Nb* punctate fluorescence throughout the cell was observed, and the combination of BIFP2::HaRxL21 and BIFC3::OBE1 yielded agglomerations which could be observed external to the nucleus (figure 4.16).

Previously, 35S::OBE1-GFP has been found to be localised to the nucleus (Saiga et al., 2008), as is HaRxL21. It is therefore surprising that the localisation of this interaction is not happening in the nucleus. A change in mRFP::OBE1 localisation in the presence of GFP::HaRxL21 was also observed by Jens Steinbrenner (figure 4.17b), however it is interesting to note when co-expressed, both proteins are present in the nucleus but do not interact there by BIFC. One possibility to be considered is that these observations are an artefact caused by the high OD of *A. tumefaciens* infiltrated into the *Nb* leaf. This is done to ensure sufficient expression of the BIFP constructs, enabling observation of *Nb* cells expressing both of the co-expressed proteins. However it must be considered that this effect is not usually observed, for example infiltration of all constructs was performed under the same conditions and fluorescing structures throughout the cytoplasm were not observed when HaRxL21 and TPL were co-expressed in BIFP vectors. To further characterise the sub-cellular structures observed when using BIFC to examine the interaction of HaRxL21 and OBE1, a titration of different *A. tumefaciens* infiltration ODs could be performed. This would enable any patterns of interaction or localisation due to high levels of protein expression to be observed, as *A. tumefaciens* mediated transformation of *Nb* results in higher levels of protein expression than would naturally occur.

The necessity of OBE1 for full *Hpa* virulence was suggested by Mukhtar et al. (2011), who showed that an OBE1 T-DNA insertion line supported reduced *Hpa* growth. The

results here (figure 4.21b) do not agree with these findings, since 2 out of 3 *A. thaliana* lines derived from SALK_075710 show no difference in susceptibility to the Col-0 GUS control line, and all three lines show no difference in susceptibility to Col-0. However, it is possible that re-localisation of OBE1 rather than complete prevention of its function could result in a contribution to virulence. It has been shown (figure 4.17b) that co-expression of OBE1 with HaRxL21 does not completely block import into the nucleus, but results in re-localisation to the cytoplasm which could be hypothesized to function in *Hpa* virulence.

To further investigate the effect of HaRxL21 on OBE, HaRxL21 constructs fused to a nuclear export signal could be expressed *in planta*. These constructs would enable the separation of effects of TPL interaction, which has been found to occur in the nucleus.

4.6.4 TOPLESS

The role of TPL and TPRs in defense of plants against phyto-pathogens is complex. TPL-mediated transcriptional repression has been found to be both extensive (Causier et al., 2012) and conserved across the plant kingdom (Causier et al., 2012). TPL has been found here to interact with HaRxL21, an interaction which has been verified by Y2H in addition to *in planta* verification using BIFC and CO-IP.

The interaction of HaRxL21 with TPL was found to be mediated by the EAR motif at the C-terminus of HaRxL21 and the CTLH domain near the N-terminus of TPL. This EAR-motif mediated interaction is well characterised in the literature; it is the same mechanism by which TPL interacts with Aux/IAA and JAZ proteins (via NINJA) (Pauwels et al., 2010), therefore holding the complex in it's inactive state and preventing transcription of IAA or JA responsive genes (Pauwels et al., 2010; Robert-Seilaniantz et al., 2011). It is interesting to note that here is an example of a phytopathogen utilising a system which is conserved in *A. thaliana*; mimicking a naturally occuring protein interaction motif. TPL seems to be a master regulator of transcription via recruitment to numerous complexes where it represses transcription via recruitment of histone acetylases (Pauwels et al., 2010; Arabidopsis Interactome Mapping Consortium, 2011; Causier et al., 2012). It is therefore possible that HaRxL21 is hijacking the host machinery and recruiting TPL into a complex to repress transcription of defence related genes, therefore promoting growth of *Hpa*.

It was also noted during Co-IP that TPL detection was much higher when co-expressed with HaRxL21, but not when TPLΔCTLH was co-expressed with HaRxL21. A similar effect was also observed when HaRxL21ΔEAR was co-expressed with TPL, meaning that TPL could often not be detected in either the input or Co-IP sample. However it seems unlikely that this was due to variation in protein expression levels in *Nb* leaves because

this effect never occurred with TPL and full length HaRxL21 were co-expressed. An alternative vector system which could be used for Co-IPs is described by Kagale et al. (2012), who show greater expression than using P19-enhanced *A. tumefaciens* mediated transformation of *Nb*. However one criticism of using a virus-based vector system such as this would be that the high expression levels may lead to increased false positives during pull downs.

The susceptibility phenotype to *Hpa* infection of *A. thaliana* plants with *TPL*, *TPR1* and *TPR4* knocked out shows that TPL and related proteins play a role in plant defense. However they are involved in such a diverse range of processes within the plant that it is difficult to pinpoint exactly what causes this. A simple explanation for the susceptibility increase observed by *in planta* expression of HaRxL21 is that it binds to TPL and titrates it away from the biological processes it usually regulates, therefore mimicking the knock-out phenotype and causing enhanced susceptibility. However in reality it is unlikely to be this straight forward due to the high protein expression levels required for this effect.

A. thaliana plants expressing HA::HaRxL21 and HA::HaRxL21ΔEAR have been generated. The protein expression levels in these plants are very low, particularly when full length HaRxL21 is expressed. It is possible that this may be due to protein degradation by the host, since RNA expression levels between lines are similar. However low protein levels are not necessarily a bad thing, as more realistic phenotypic changes in response to the action of the effector protein can be observed when the protein is not over-expressed to a very high level. It was also noticed that in Figures 4.26d and 4.27, susceptibility was correlated with protein expression level; HA::HaRxL21ΔEAR-1 shows the highest protein expression and the least susceptible plant phenotype. This is possibly due to ER stress and the protein unfolded response (Vitale and Boston, 2008; Deng et al., 2013). When looking at *HaRxL21* expression levels by qRT-PCR, similar levels are observed in HA-tagged and previously used un-tagged HaRxL21 plants, which suggests that these plants may also exhibit low protein concentrations.

When directly compared, *A. thaliana* plants expressing HA::HaRxL21 were more susceptible to *Hpa* than plants expressing HA::HaRxL21ΔEAR (Figure 4.27). However no reproducible differences were observed between either HA::HaRxL21 or HaRxL21ΔEAR with controls (Col-0 and HA::GFP), which is unusual since enhanced susceptibility has previously been reported for 35S::HaRxL21-expressing *A. thaliana* lines. It is possible that this is due to experimental conditions, since the line 'F1/3' which is used as a control for enhanced susceptibility was not showing its expected phenotype. The reduced germination rate observed in HA::HaRxL21 lines compared to controls may have also impacted their susceptibility, since very few plants germinated per module therefore affecting the

local humidity and infection by *Hpa*.

An alternative hypothesis is that the HA tag at the N-terminus of HaRxL21 is shielding a previously un-described interaction, therefore preventing the action of the effector protein. Is it possible that if HaRxL21 is recruiting TPL to a site of transcriptional initiation, an interaction at the N-terminus of the protein is mediating interaction with a host target such as a transcription factor. Future work will therefore be to continue characterisation of these plant lines and to generate plants expressing HA::HaRxL21ΔEAR in the absence of a N-terminal tag, thus allowing any susceptibility differences to full length HaRxL21 to be examined.

4.6.5 Other EAR domain containing effector proteins

Screening of *Hpa* RxL and RxLL effector candidates for the EAR motif yielded more results than expected, given that HaRxL21 was the only effector identified by Mukhtar et al. (2011) to interact with TPL. However HaRxL21 is unique in that it is the only effector to contain an EAR motif at the absolute C-terminus, there is only one amino acid between the EAR motif and the stop codon. This may be the reason that none of the other RxLs and RxLLs tested were found to interact with TPL using Y2H. However not all EAR-motif containing RxLs/RxLLs were cloned and therefore were able to be tested for TPL interaction. One possible candidate is HaRxL48 which, like HaRxL21 has been found to be nuclear localised and contains an EAR motif near the C-terminus. HaRxLL6 and HaRxLL39 also contain EAR motifs within a few amino acids of the C-terminus of the protein.

In the future, it may be worth testing for interaction of other EAR motif containing effectors with TPL using BIFC, since it has been shown by HaRxL21 Cala2 that not all interactions can be seen by Y2H. These data also highlight the fact that alleles of effectors may show different interacting partners and if only the Cala2 allele of HaRxL21 had been included by Mukhtar et al. (2011) then an interaction with TPL would not have been picked up. Using alleles of effectors may also give us further insight into their evolution as the capability for protein-protein interactions which do not provide a functional benefit may be lost.

In addition, it is interesting to note that the motif LRLFL is present in the CRN domain of *Hpa* CRINKLER effectors. Another example of a pathogen effector protein utilising the EAR motif is the XopD; a type III effector from *Xanthomonas campestris* with homologues in the *Acidovorax* and *Pseudomonas* genera (Kim et al., 2011). It is therefore

plausible that more than one effector from *Hpa* or from other oomycete pathogens such as *Pi* may utilise this system of hijacking host gene regulation.

Chapter 5

Transcriptional changes induced by HaRxL21

5.1 Introduction

By studying the transcriptional changes brought about during pathogen infection, we have been able to better understand how plants defend themselves. In addition, we've gained insights as to how pathogens manipulate host defense responses. Many of the downstream responses to PTI and ETI (as discussed in sections 1.3 and 1.5) lead to transcriptional changes involved in hormone biosynthesis and transcription of pathogenesis-related genes. It is also well documented that multiple stress responses in plants both share some transcriptional pathways and regulate each other (Cheong et al., 2002; Robert-Seilaniantz et al., 2011).

Recently, high resolution data sets have been generated to document transcriptional responses to pathogen infection, therefore allowing for a more detailed analysis of transcriptional events during pathogen colonisation. For example the *A. thaliana* defense response to *B. cinerea* has been studied in detail (Windram et al., 2012) (Figure 5.1). It can be observed that by 14 hours post infection the plant is responding transcriptionally; genes involved in hormone biosynthesis are up regulated and genes involved in 'normal' cell functioning such as chlorophyll biosynthesis are down regulated. However one limitation of this data set is that transcriptional changes cannot be distinguished between those which form part of the host defense response and those induced by pathogen effector proteins.

Thilmony et al. (2006) have provided information about manipulation of host transcription by the pathogen *Pseudomonas syringae* pv. *tomato* DC3000. The advantage of this

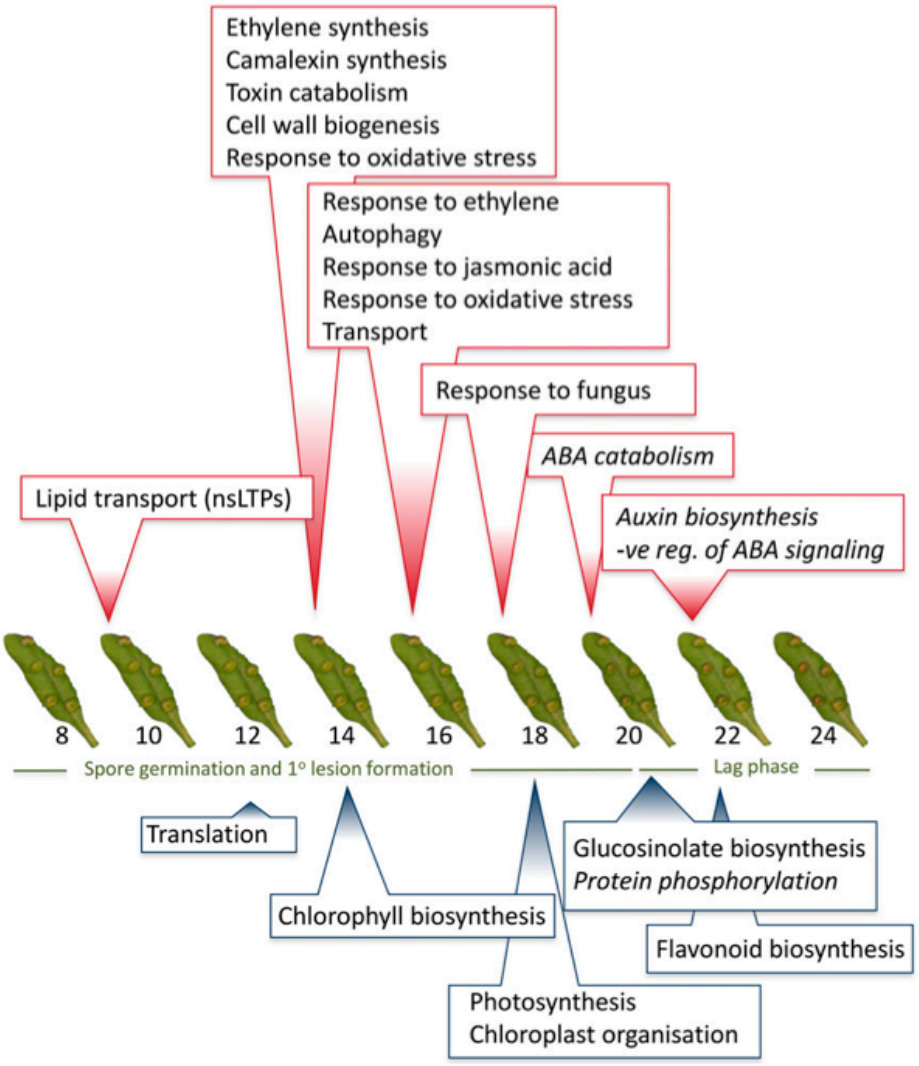


Figure 5.1: Genetic changes in response to *B. cinerea* infection of *A. thaliana*.
Time course showing over-represented GO terms in differentially expressed gene clusters in response to *B. cinerea* infection of *A. thaliana* leaves. Reproduced with permission from Windram et al. (2012).

data set is that it also details transcriptional responses in plant tissue infected with the *P. syringae hrpA* mutant (compromised in its ability to deliver type III effector proteins (Gopalan et al., 1996; Collmer et al., 2000)) and the *P. syringae* DC3118 COR$^-$ mutant (defective in production of the virulence factor coronatine (COR) (Mitchell, 1982)). This approach therefore allows separation of transcriptional changes in 'normal' host defense responses from those resulting from the action of pathogen effectors, giving us greater understanding of how pathogens manipulate their hosts.

5.1.1 Pathogen effector proteins which manipulate host transcription

Since so many plant defense responses are transcriptionally regulated, manipulation of host transcription is a 'logical' target for pathogen effector proteins. Described examples include the *Xanthomonas campestris* effector XopD which alters host transcription by targeting the transcription factor MYB30 (Kim et al., 2008; Canonne et al., 2011) and transcription activator-like effector (TALe) proteins secreted by the genus *Xanthomonas* which bind to and activate host promoters (Römer et al., 2010), for example the effector AvrXa27 (Gu et al., 2009). The *P. syringae* effector HopU1 utilises an alternative mechanism, affecting RNA metabolism and therefore transcription of defense genes (Fu et al., 2007).

A plethora of effectors from *Hpa* have been identified which localise to the host nucleus (Caillaud et al. (2011) and Laura Lewis, personal communication). It is therefore likely that the function of these proteins is to manipulate host transcription. HaRxL21 localises to the host nucleus and has been shown to interact with the known transcriptional co-repressor TOPLESS (TPL) (section 4.3.1), suggesting a role in manipulation of host transcriptional responses to pathogen attack. In order to establish the effect of HaRxL21 on host transcription, *A. thaliana* plants of the ecotype Col-0 constitutively expressing HaRxL21 under the control of the 35S promoter were used (HaRxL21a-c; detailed in section 2.1.7). Using these plants it is possible to examine how gene expression levels differ from wild type both in the absence of pathogen challenge and during infection. However, one disadvantage of these plant lines is that due to constitutive expression, downstream and developmental effects may be observed and the direct targets of HaRxL21 action cannot be elucidated by this method.

5.1.2 Aims

The aim of the research here is to establish whether HaRxL21 has an effect on host transcription, particularly during defense to pathogens.

5.2 *Botrytis cinerea* Microarrays

It was previously observed that *in planta* expression of HaRxL21 causes enhanced susceptibility to *B. cinerea* (Figure 3.8). To investigate whether this was due to changes at the transcriptional level, microarrays were performed on two independently transformed lines constitutively over-expressing HaRxL21 (HaRxL21a and HaRxL21b) and Col-0. RNA samples were extracted from un-infected plants and 22 hours-post infection with *B. cinerea*. Twenty two hours post infection was chosen as a time point by which transcriptional changes in response to infection would usually have occurred (Figure 5.1), therefore allowing suppression of these responses by HaRxL21 to be observed.

Transcriptional information was obtained by performing CATMA (Complete Arabidopsis Transcriptome MicroArray) microarrays (Crowe et al., 2003; Hilson et al., 2004). These arrays are two-colour and therefore a loop design was used. This means that every possible comparison was paired together on an array slide, thus allowing comparison of relative gene expression between the treatments. When designing the experiment, the need for dye swaps (every sample labelled by both Cy3 and Cy5) and technical replicates to account for variation between dyes and between array slides was taken into account, in addition to biological replicates. The final design (Andrew Mead, University of Warwick, personal communication) was a loop design and consisted of twenty four microarray slides (described in section 2.2.15.4 and Table 2.7).

5.2.1 Data quality assessment

After intra and inter-array normalisation, the quality of data was assessed. A box plot of average array intensity is shown in Figure 5.2. It can be observed that even after normalisation there is some variation in intensity between arrays, however the technical replicates and dye swaps in the experimental design should account for this.

5.2.2 Summary of differentially expressed genes

Data analysis was performed using MAANOVA (Microarray analysis of variance (Wu et al., 2008) to determine genes which were differentially expressed (as described in section 2.2.15.6). MAANOVA uses an analysis of variance (ANOVA) to determine for each probe whether variation between experimental conditions is greater than variation between replicates, generating an F statistic for each probe. P-values are then generated (a

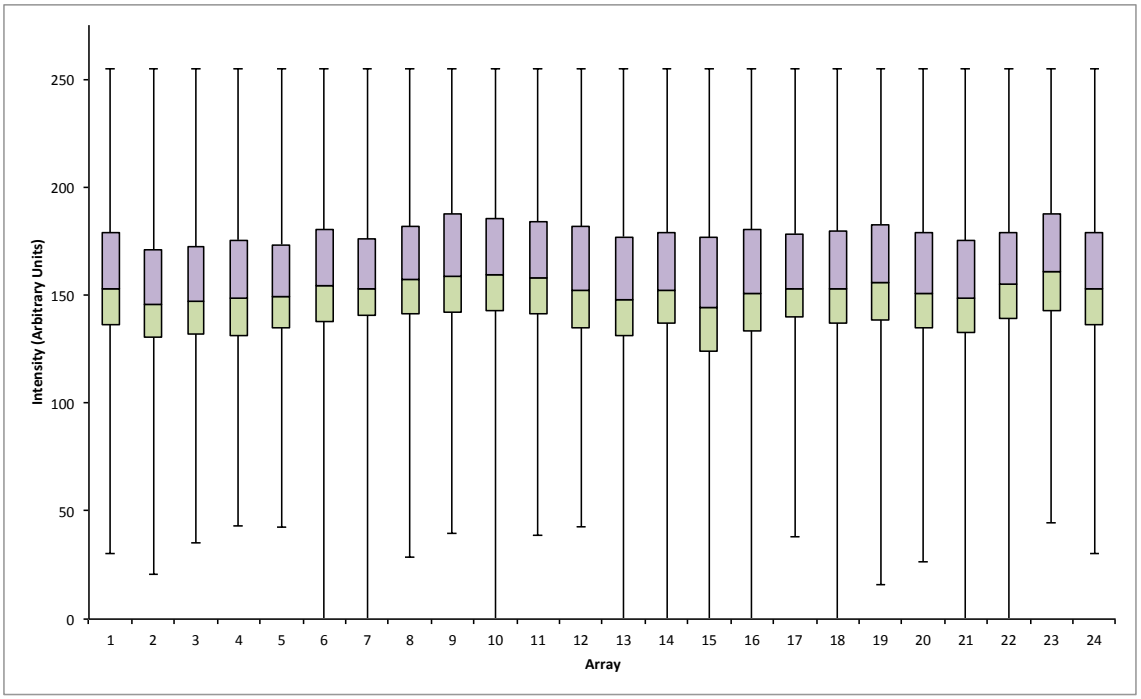


Figure 5.2: Microarray intensity after intra and inter-array normalisation.
Box plot showing probe intensity across the 24 microarray slides used in this experiment.

low P-value corresponds to a high F-statistic) which were used to identify significantly differentially expressed genes.

A summary of differentially expressed genes for each pairwise comparison is shown in Table 5.1; data shown uses 'Adaptive' false discovery correction (Benjamini and Hochberg, 2000). Three different P-value cut offs are shown here and a P-value cut off of 0.05 was chosen for subsequent analysis. This was because the subset of genes differentially regulated at the 95 % confidence interval showed some differential regulation for all comparisons while still retaining statistical significance.

It can also be observed that in uninfected tissue HaRxL21a has fewer differentially expressed genes than HaRxL21b when compared to the Col-0 wild type (200 and 1106 respectively when $P<0.05$). The largest differences in gene expression can be observed between the infected and uninfected samples for each *A. thaliana* line investigated (approximately 15,000 genes), however there are very few genes differentially regulated between HaRxL21a and b with Col-0 after 22 hours of infection with *B. cinerea* (3 and 107 genes respectively). It is also noteworthy that in both infected and uninfected tissue there is a higher number of differentially expressed genes between the two independently transformed 35S::HaRxL21 lines than between HaRxL21a and Col-0 (Table 5.1).

Differential gene expression between the six treatments can also be represented as a heat

Table 5.1: Number of differentially expressed genes at different significance levels

Comparison	P<0.01	P<0.05	P<0.1
Uninfected HaRxL21a vs Uninfected Col-0	57	200	434
Uninfected HaRxL21b vs Uninfected Col-0	454	1106	1728
Uninfected HaRxL21a vs Uninfected HaRxL21b	126	458	897
Infected HaRxL21a vs Infected Col-0	0	3	49
Infected HaRxL21b vs Infected Col-0	6	107	284
Infected HaRxL21a vs Infected HaRxL21b	0	10	55
Uninfected HaRxL21a vs Infected HaRxL21a	11611	15210	17568
Uninfected HaRxL21b vs Infected HaRxL21b	11539	14769	16967
Uninfected Col-0 vs Infected Col-0	11029	14968	16593

map (Figure 5.3), in which red indicates low gene expression and green indicates high expression. It can be observed that the majority of changes can be observed between infected and uninfected plants, however there are clearly some differences in expression which can be seen between Col-0 and both HaRxL21 expressing lines in uninfected tissue.

5.2.3 Differentially expressed genes prior to infection

5.2.3.1 Differentially expressed genes in *both* HaRxL21a and HaRxL21b

In uninfected tissue, taking the subset of genes which are differentially expressed in *both* HaRxL21a and HaRxL21b compared to wild type (Table 5.1), there are 138 differentially expressed genes at a P-value cut off of 0.05; 65 up-regulated and 73 down-regulated.

'Gene Ontology' (GO) terms (Reference Genome Group of the Gene Ontology Consortium, 2009) are a tool which can be used to characterise subsets of genes. BINGO (Biological Network Gene Ontology) (Maere et al., 2005) is java-based plugin for Cytoscape which will identify over-represented GO terms in a list of genes. BINGO analysis of the 138 differentially regulated genes show enrichment of GO terms involved in response to stimuli, specifically water stress and hormone (ABA) stimulus (Figure 5.4).

Interestingly, if you perform BINGO analysis on the 73 down-regulated genes, there are only three significantly over-represented categories; GO=IDs 16759 (cellulose synthase activity), 30312 (external encapsulating structure) and 5618 (cell wall). In contrast, performing BINGO analysis on the 65 up-regulated genes shows an over-representation of GO-IDs involved in response to stimuli; for example GO-IDs 9725 (response to hormone stimulus), 9719 (response to endogenous stimulus), 9737 (response to ABA stimulus),

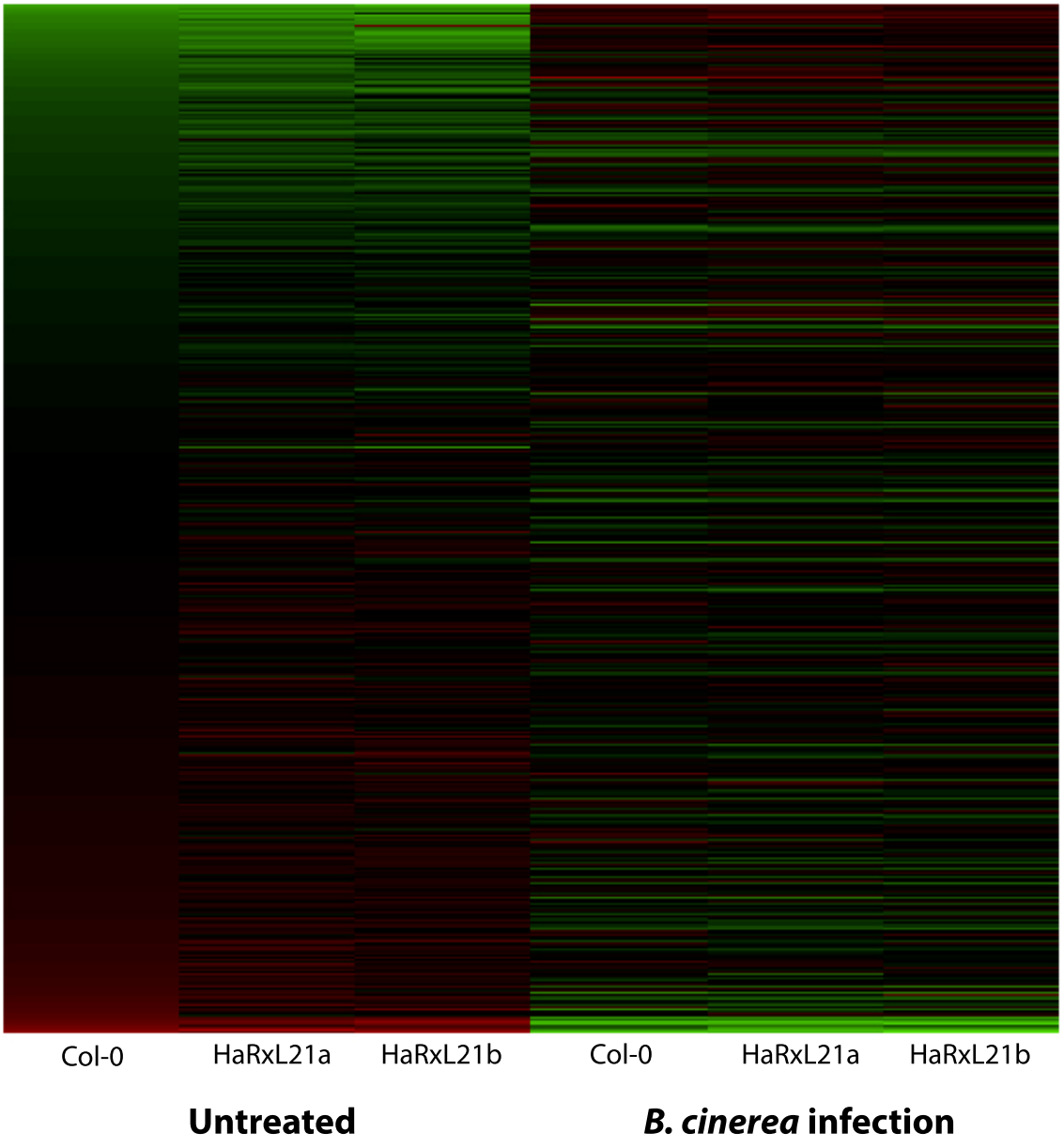


Figure 5.3: Differential gene expression across all treatments.
Expression values across all genes between untreated and *B. cinerea* infected samples of Col-0, HaRxL21a and HaRxL21b. Data shown are the relative expression values fitted when variance from other sources such as array slides, dye and biological replicates has been removed. Values range from red (low expression) to green (high expression). Gene IDs have been ordered in accordance with expression levels in uninfected Col-0.

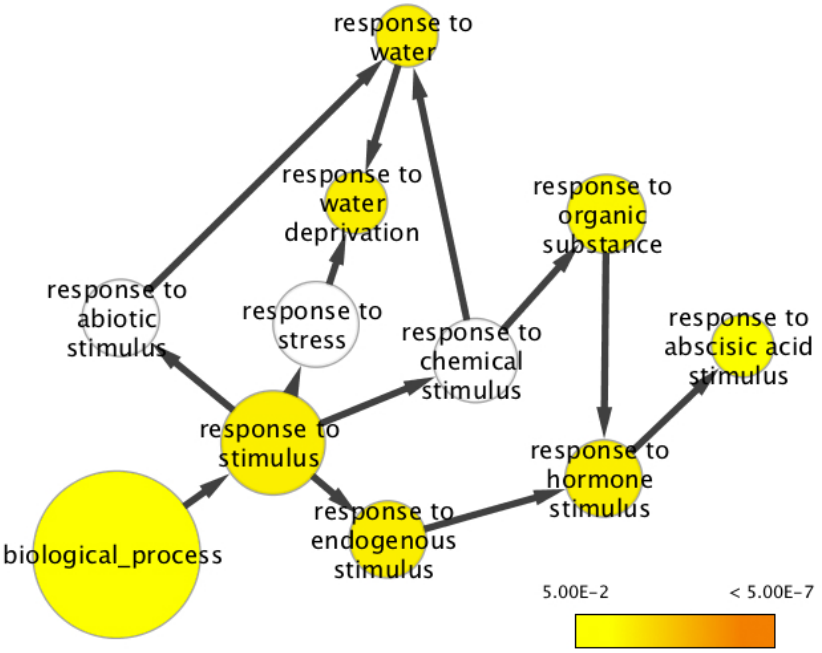


Figure 5.4: Enriched GO terms in differentially expressed genes in HaRxL21a and HaRxL21b compared to Col-0.
Enriched GO terms in differentially expressed genes in both HaRxL21a and HaRxL21b compared to Col-0. BINGO visualisation is used to show over-represented GO terms after Benjamini and Hochberg (2000) false discovery rate correction.

9414 (response to water deprivation), 9651 (response to salt stress), 9628 (response to abiotic stimulus) and 9755 (hormone-mediated signalling pathway). Of these, up regulated genes known to be involved in response to stress include the transcription factor *MYB96*, the ethylene response factor *ERF72* (Li et al., 2008), the gene encoding the tubby-like protein TLP2 (Reitz et al., 2012), the ABA-responsive element ABF3 (Oh et al., 2005) and the receptor like protein RLP32 which is up regulated upon drought and heat stress (Rizhsky et al., 2004).

5.2.3.2 Differentially expressed genes using mean gene expression in HaRxL21a and HaRxL21b

An alternative approach uses the mean gene expression values for the two lines HaRxL21a and HaRxL21b. This approach allows for the inclusion of genes which may fall just outside of the cut offs when considering the lines independently, but the combined probability of them being differentially expressed in both lines suggests they may be biologically relevant. This approach results in 686 genes which are differentially regulated (P<0.05); 386 down regulated and 300 up regulated.

Down-regulated genes

BINGO analysis (Maere et al., 2005) was performed on the 386 down-regulated genes. The result was a variety of over-represented GO-terms including response to stress, biotic stimuli and abiotic stimuli. There were also over-represented GO-terms involved in cell structure and cellular processes such as translation (Figure 5.5). In total, there were 76 genes identified by the GO-term 'response to stimulus' and 49 genes identified by the GO-term 'response to stress'. Down regulated genes categorised by the GO-term'response to biotic stimulus' include the pathogenesis related gene *PR1*, Downy Mildew Resistant 6 (*DMR6*) and 2 members of the heat shock protein 70 family. Interestingly, down regulated genes also include *MBP2*, *MBP1*, *VSP1* and *PDF1.2*, all of which are up-regulated in response to MeJA treatment (Pauwels et al., 2010). Also down regulated are the histone H2A proteins HTA11 and HTA13, and the MYB transcription factors MYB111, MYB103 (also called MYB80) and MYB75.

Upregulated genes

BINGO analysis was performed on the 300 up-regulated genes (Figure 5.6). Results here clearly show over-representation of GO-terms involved in response to hormone stimuli, specifically response to ABA stimulus and ABA signalling, therefore suggesting that genes involved in response to ABA are up regulated.

Up regulated genes which were labelled with the GO-term 'response to abscisic acid stimulus' included the abscisic acid responsive element-binding factors *ABF1* and *ABF3*, the transcription factors *MYB96* and *MYB-LIKE 2*, genes encoding two homeobox proteins ATHB-7 and ATHB-12, the receptor-like kinase RPK1 (which is usually regulated by ABA), ABI five binding protein AFP1 and two homologues of an ABA- and stress-inducible gene from barley; HVA HOMOLOGUE D and E. Up regulated genes labelled with the GO-term 'response to hormone stimulus' included ABA responsive proteins, in addition to an ethylene response factor (*ERF72*), gibberellin-regulated genes *GASA1* and *GASA6*, and genes encoding proteins involved in auxin signalling (IAA28 and TIR1) and biosynthesis (TAA1).

In addition, the histone H2A proteins HTA4 and HTA12 and several NF-Y transcription factors were found to be up regulated; NF-YC12, NF-YA9 and NF-YB5.

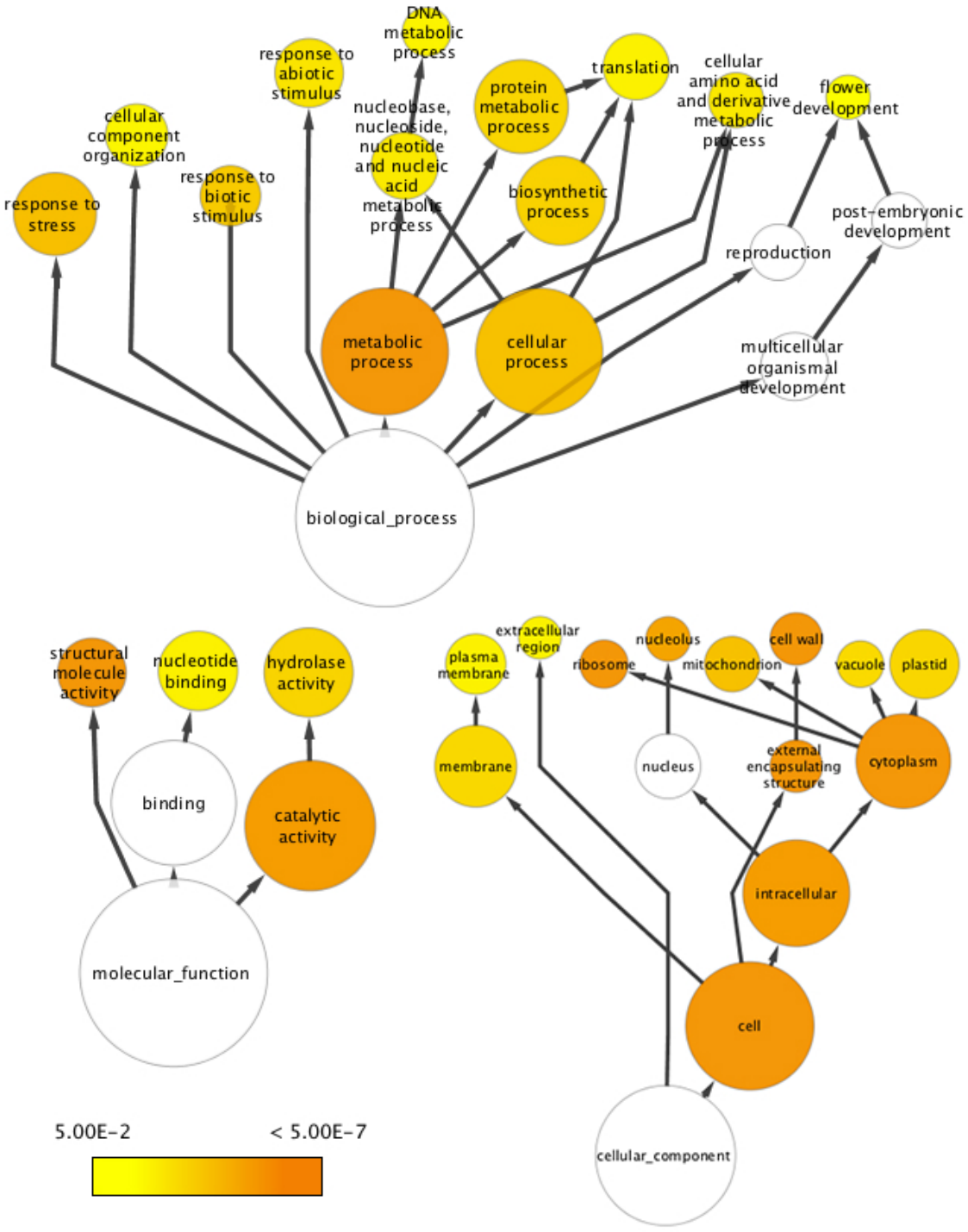


Figure 5.5: GO-terms over-represented in down regulated genes in HaRxL21a and HaRxL21b plants compared to Col-0

BINGOSlimPlants categories over-represented in the 386 genes which were down regulated using the average expression in HaRxL21a and HaRxL21b compared to Col-0 wild type. Colouration indicates significance of over-representation as indicated by key.

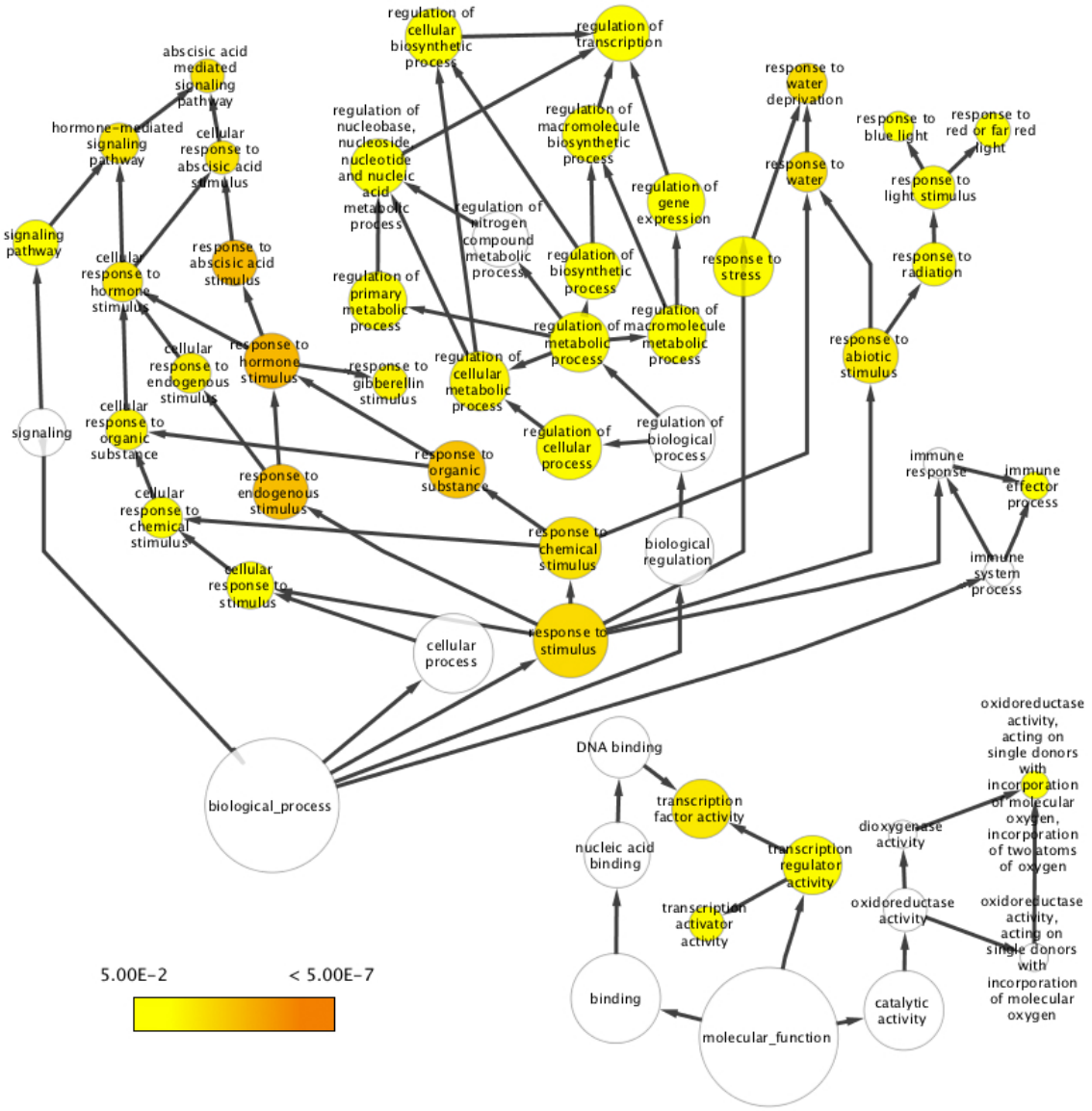


Figure 5.6: GO-terms over-represented in up regulated genes in HaRxL21a and HaRxL21b plants compared to Col-0

BINGOSlimPlants categories over-represented in the 300 genes which were up regulated using the average expression in HaRxL21a and HaRxL21b compared to Col-0 wild type. Colouration indicates significance of over-representation as indicated by key.

5.2.4 Differentially expressed genes after *B. cinerea* infection

In infected tissue, taking the subset of genes which are differentially expressed in *both* HaRxL21a and HaRxL21b compared to wild type (Table 5.1), the overlap between the two lines is 2 genes (P<0.05). These genes; AT4G11320 (cysteine protease 2 (CP2)) and AT1G64520 (regulatory particle non-ATPase 12A (RPN12a)) were both down regulated relative to wild type expression levels.

Using the Arabidopsis eFP browser (Winter et al., 2007), RPN12a does not show differential expression in response to biotic stress. CP2 shows up-regulation in response to virulent (but not to avirulent) *P. syringae* but, interestingly, not to *B. cinerea* infection (Figure 5.7). In the data obtained here, CP2 shows down regulation upon *B. cinerea* infection in Col-0. In HaRxL21a and b expression of this gene is down regulated prior to infection, which means that although it is less down regulated upon infection, the resulting expression in infected tissue is lower than wild type.

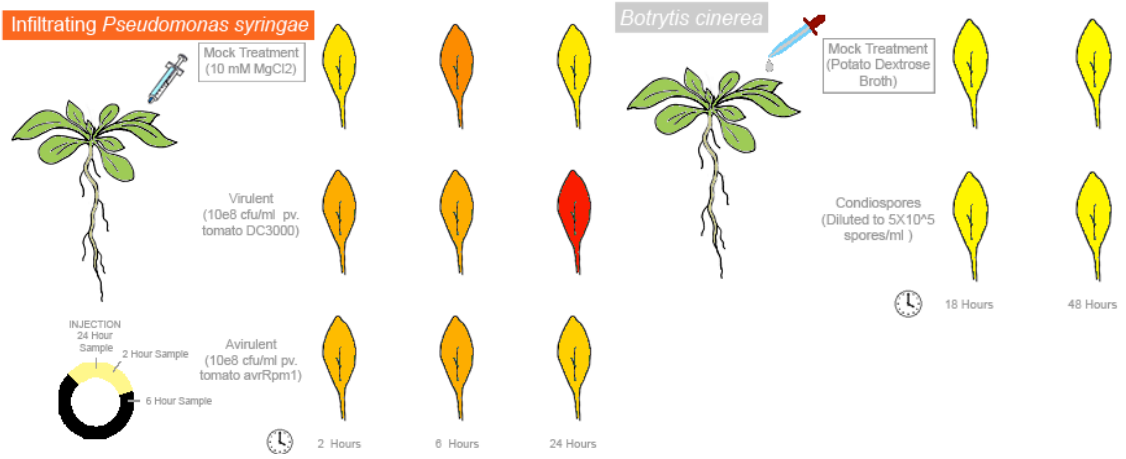


Figure 5.7: Cysteine protease 2 (AT4G11320) expression in response to biotic stimulus.
Cysteine protease 2 expression in response to *P. syringae* and *B. cinerea*. From the Arabidopsis eFP browser; Winter et al. (2007).

Using mean expression values between HaRxL21a and HaRxL21b, 59 genes were found to be differentially regulated after infection; 20 up regulated and 39 down regulated. Using BINGOSlimPlants, no GO-terms were found to be enriched in those genes which were up regulated. In the 39 down regulated genes, significant enrichment was found for the GO-IDs 9628 (response to abiotic stimulus), 9719 (response to endogenous stimulus) and 8289 (lipid binding).

5.2.5 Differentially expressed genes in response to *B. cinerea* infection

Previously, differentially expressed genes before or after infection have been considered (depicted by Figure 5.8a). How genes respond to infection was therefore taken into account to determine whether HaRxL21 was suppressing activation of any defense responses.

Genes which were significantly differentially expressed upon infection were identified within MAANOVA using a contrast matrix, which takes into account values before and after infection then performs pairwise comparisons to determine if genes respond differently to treatment.

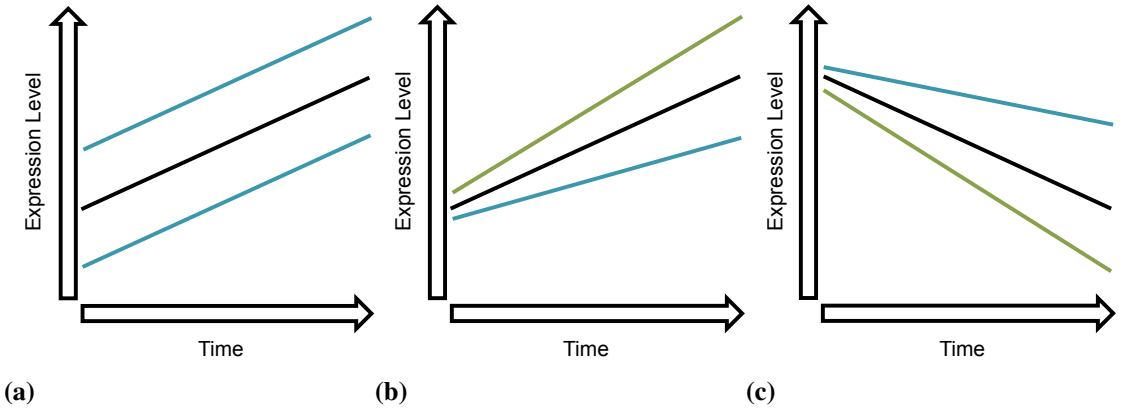


Figure 5.8: Differential gene expression patterns in microarray data.
Patterns of differential gene expression considered in microarray data; (a) genes which are expressed differentially to wild type level (black) irrespective of expression, (b) genes normally up regulated in response to infection which respond less (blue) or more (green). (c) Genes normally down regulated in response to infection which respond less (blue) or more (green).

In total, there were 279 genes which responded significantly differently to *B. cinerea* treatment between Col-0 and HaRxL21a and b ($P<0.05$). Of these, 125 genes responded more (green in Figure 5.8b and 5.8c) and 154 genes responded to a lesser extent in HaRxL21a and b compared to Col-0 (blue in Figure 5.8b and 5.8c).

Of the genes which responded less in HaRxL21a and b compared to Col-0, 63 were genes which were up regulated in Col-0 in response to infection (Figure 5.8b, blue). To eliminate genes which have little biological significance due to small expression changes, genes were considered if they showed a fold change of greater than 2 in Col-0 upon infection. Ten genes met these criteria and are shown in Table 5.2. Of these 10 genes, AT1G02850, AT1G77450, AT1G08230, AT1G62570 have been previously shown to be up regulated upon *B. cinerea* infection (Winter et al., 2007). AT3G61890, AT4G21510, AT1G80130,

AT1G32900 and AT2G47180 were not found to show up regulation upon *B. cinerea* infection and no expression data was available for AT1G53480.

Table 5.2: Genes which show reduced up regulation in response to *B. cinerea* infection in HaRxL21a and b compared to Col-0.

ATG	Name	Description	FC Col-0	FC HaRxL21a	FC HaRxL21b	FC HaRxL21a and b / Col-0	P-value
AT1G02850	*BGLU11*	BETA GLUCOSIDASE 11.	2.889308835	1.880611866	1.905920129	0.655266053	0.003107055
AT1G77450	*anac032*	Arabidopsis NAC domain containing protein 32.	2.67640438	2.000212787	2.087330089	0.763625801	0.029075685
AT1G08230	*AtGAT1*	High affinity gamma-aminobutyric acid (GABA) transporter.	2.189090751	1.464220171	1.434886351	0.662171388	0.029075685
AT3G61890	*ATHB-12*	ARABIDOPSIS THALIANA HOMEOBOX 12.	2.167142156	0.938628383	1.727822463	0.615199801	0.03421274
AT1G62570	*FMO_GS-OX4*	FLAVIN-MONOOXYGENASE GLUCOSINOLATE S-OXYGENASE 4.	1.79220513	0.597838195	0.611838963	0.337482897	0.023528143
AT4G21510	*ATFBS2*	F-BOX STRESS INDUCED 2.	1.529525977	0.90449145	0.839722499	0.570181211	0.03262563
AT1G80130	*TPR-like*	Tetratricopeptide repeat (TPR)-like superfamily protein.	1.236748261	0.55259063	0.034531363	0.237365199	0.030568716
AT1G53480	*ATMRD1*	MTO 1 RESPONDING DOWN 1.	1.216752467	-0.054646034	0.304844982	0.102814235	0.011837762
AT1G32900	*GBSS1*	GRANULE BOUND STARCH SYNTHASE 1, UDP-Glycosyltransferase superfamily protein.	1.213492159	0.560892632	0.187906881	0.308530841	0.049378754
AT2G47180	*ATGOLS1*	Arabidopsis thaliana galactinol synthase 1.	1.148409962	0.611501997	0.193031328	0.350281411	0.006618161

Genes which responded significantly differently upon infection between HaRxL21a/b and Col-0 which showed greater than 1 ($\log_2$) fold up regulation in Col-0. Descriptions from The Arabidopsis Information Resource (TAIR). FC = Fold change. P-values are shown for significant difference after adaptive false discovery rate correction (Benjamini and Hochberg, 2000).

5.3 Verification of Microarray Results

5.3.1 JA Root Length Inhibition

When looking at genes which are down-regulated in HaRxL21-expressing *A. thaliana* plants compared to wild type (prior to *B. cinerea* infection), a down regulation in marker genes for JA signalling (such as *PDF1.2*) was observed. JA signalling is known to cause root growth inhibition (Staswick et al., 1992) and therefore by examining root length in the presence and absence of MeJA it is possible to test whether plants have a decreased sensitivity to JA.

Seedlings of *A. thaliana* lines HaRxL21a, b and c (in addition to Col-0 and Col-0 GUS controls) were grown on MS plates (method described in section 2.2.1.2) containing MeJA and root length was measured. It was observed that there was variation in initial root length and so root growth inhibition on 10 μM MeJA plates was calculated as a percentage of root length in the absence of MeJA. It was found that *in planta* expression of HaRxL21 caused a significant reduction in root growth inhibition compared to Col-0 in lines HaRxL21a and HaRxL21c (Figure 5.9).

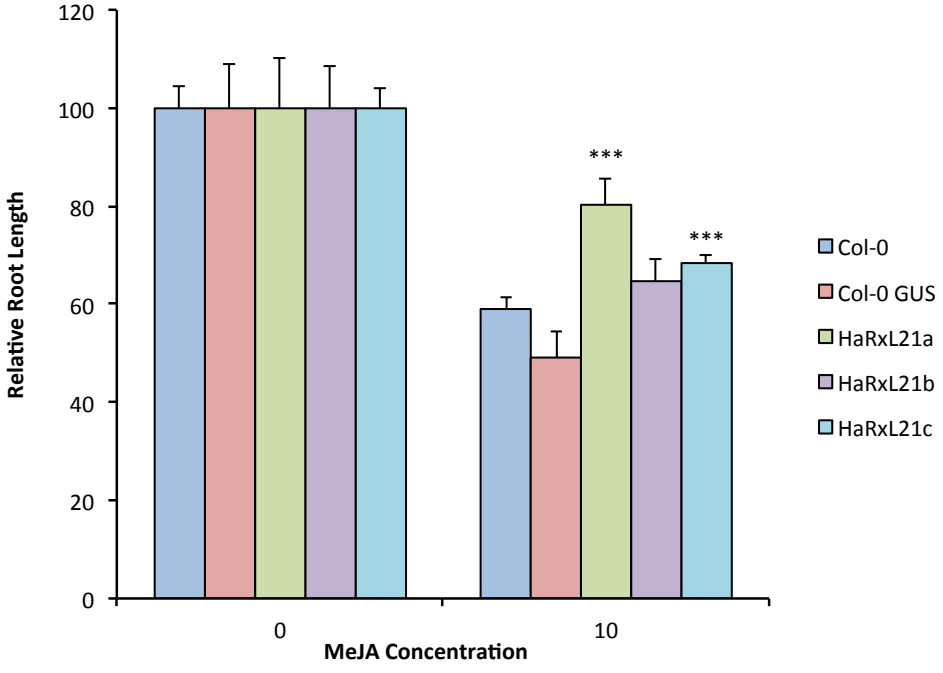


Figure 5.9: HaRxL21 suppresses MeJA mediated root growth inhibition.
Root length of HaRxL21a, b and c compared to controls Col-0 and Col-0 GUS. Root length on MS plates containing 10 μM MeJA is expressed as a percentage of root length on plates containing no MeJA. Error bars show standard error and significant differences to Col-0 are shown; *** = P<0.001.

5.3.2 ABA Germination

HaRxL21 has been found to show up regulation in genes involved in ABA signalling (Figure 5.6) and therefore it was investigated whether any physiological responses to ABA were enhanced in plants expressing HaRxL21. The presence of ABA is known to inhibit germination and therefore germination on media containing ABA can therefore be used to test whether ABA sensitivity is altered (Koornneef et al., 1984; Finkelstein, 1994). *A. thaliana* seeds were plated onto MS plates containing 0 mM ABA or 0.5 mM ABA (as described in section 2.2.1.2) and the percentage germination recorded. Data were analysed using a paired T-test between plant lines sown on the same plate. In all cases, 100 % germination was observed on plates containing no ABA. It was found that in the presence of 0.5 mM ABA, HaRxL21b showed a significant reduction in germination compared to Col-0 and HaRxL21c showed a reduction in germination although this was not significant (P=0.054) (Figure 5.10).

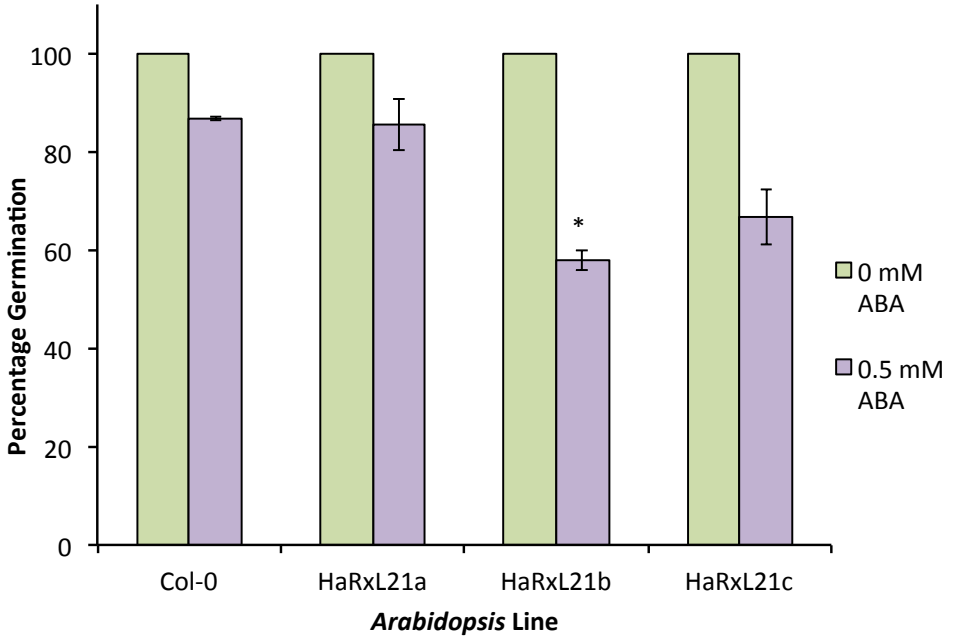


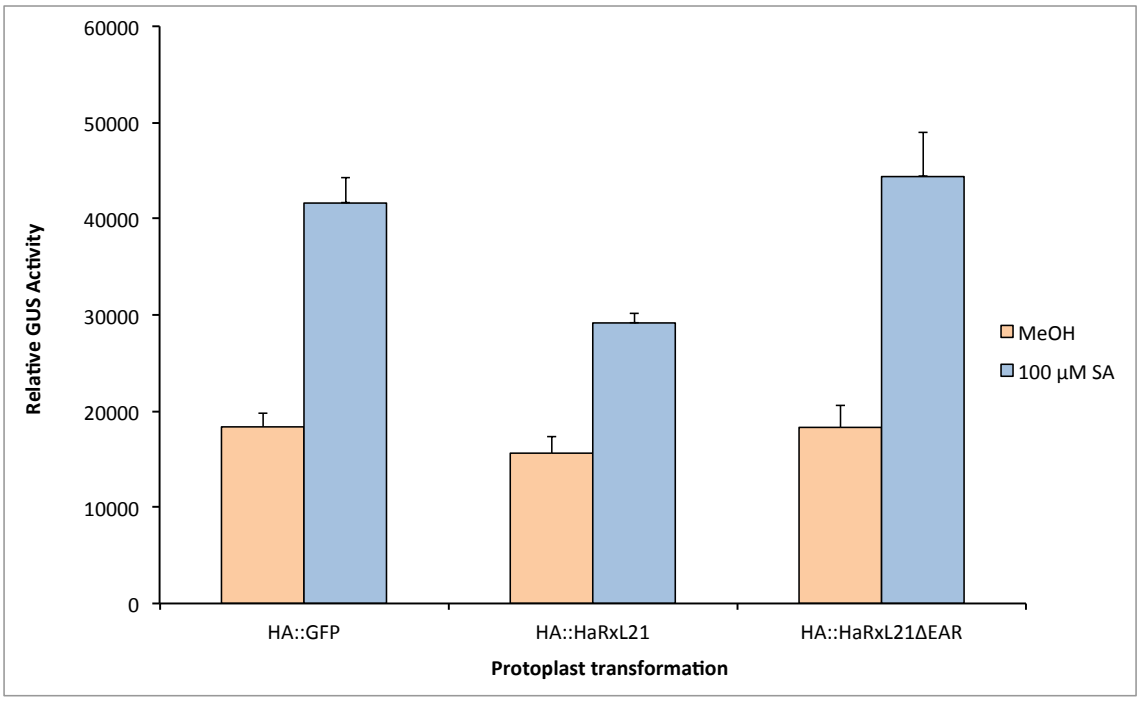
Figure 5.10: HaRxL21b shows decreased germination in the presence of ABA compared to Col-0.
Percentage germination across 40 seeds on 2 plates containing each of 0 mM and 0.5 mM ABA. HaRxL21a-c are compared to Col-0 control. Error bars show standard error and significant differences to Col-0 are shown; * = P<0.05.

5.3.3 *Arabidopsis thaliana* mesophyll protoplasts

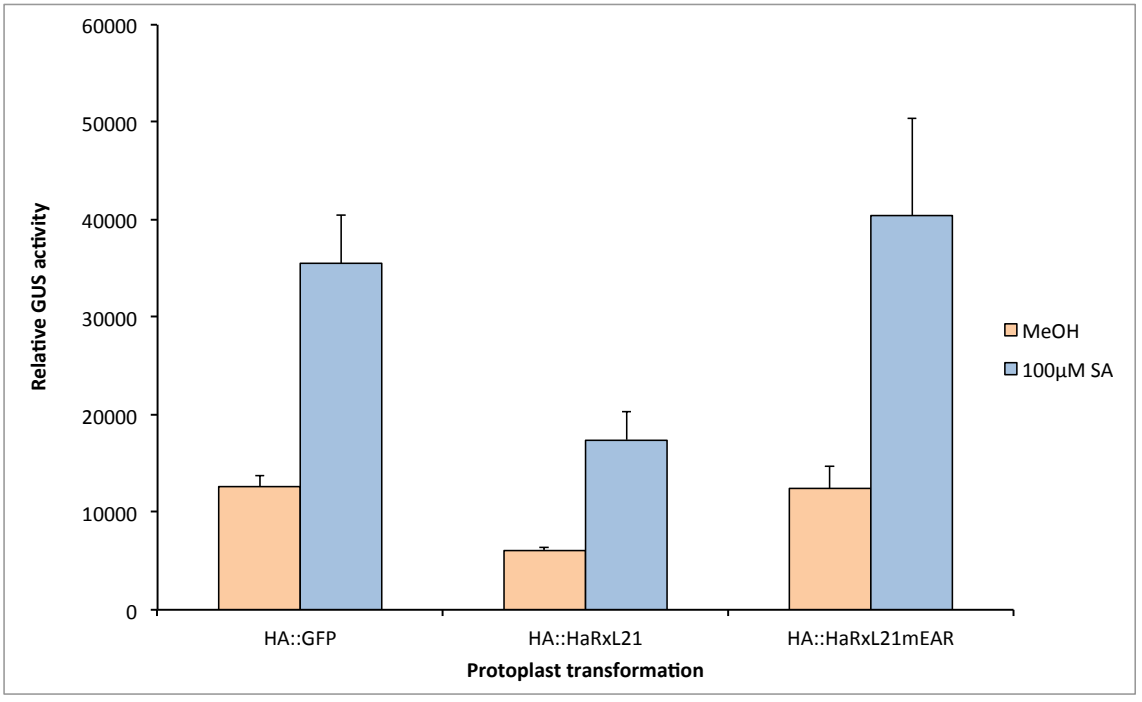
A. thaliana plants expressing HaRxL21 show enhanced susceptibility to *Hpa* (Figure 3.5a). Defense to biotrophic pathogens such as *Hpa* is mediated through signalling induced by the phytohormone SA. The reporter gene *PR1* is induced upon SA treatment and has been observed to show a reduced basal expression level in plants expressing HaRxL21 (compared to wild type Col-0) prior to *B. cinerea* infection. To determine whether this observation translates into reduced *PR1* activation upon SA induction, a reporter system using Col-0 PR1::GUS plants can be used. This assay works by measuring GUS activity upon SA treatment in *A. thaliana* protoplasts generated from this plant line (He et al., 2007); less GUS activity corresponds to reduced induction of *PR1*.

A. thaliana protoplasts were generated from Col-0 PR1::GUS plants and transfected with HA::HaRxL21, HA::HaRxL21ΔEAR or HA::GFP (method described in section 2.2.17). HA::HaRxL21ΔEAR was included to establish whether any effects observed were due to interaction with TPL, since deletion of the EAR motif removes interaction with TPL (Figure 4.6). Protoplasts were also transfected with 35S::Luciferase control which is used as a measure of transformation efficiency; by adjusting GUS measurements to luciferase measurements it is possible to account for varying protoplast transformation efficiency (as described in Yoo et al. (2007)).

It was observed that HaRxL21 caused a reduction in *PR1* activation compared to a GFP control, and that this effect was abolished when the EAR domain of HaRxL21 was deleted. The combined results from four experiments (two of which were performed by Daniel Tome) are shown in Figure 5.11a. The experiment was then repeated using a clone of HaRxL21 in which the Leu residues in the EAR motif were mutated to Phe (designated HaRxL21mEAR). It was observed that full length HaRxL21 was able to suppress *PR1* activation (as previously observed) but HaRxL21mEAR was not (Figure 5.11b).



(a)



(b)

Figure 5.11: HaRxL21 suppresses *PR1* activation upon SA treatment in *A. thaliana* protoplasts in an EAR domain dependent manner.
A. thaliana protoplasts were treated with either MeOH or 100μM Salicylic Acid in MeOH. *PR1* activation was measured through fusion to the GUS protein, activity of which was measured. Full length HaRxL21 was compared to (a) HaRxL21ΔEAR and (b) HaRxL21mEAR. The combined results of four independent experiments performed by Daniel Tome and Sarah Harvey are shown for each. Error bars display standard error, n=4.

5.4 Salicylic Acid Treated Arrays

To investigate whether *in planta* expression of HaRxL21 compromised signalling and subsequent responses to SA, 21 day old *A. thaliana* seedlings were sprayed with SA. Twenty four hours post spraying, samples were taken and SA induction was verified by reverse-transcription (RT) PCR of Col-0 samples to confirm *PR1* induction (method and primers described in section 2.2.16.2). Induction of *PR1* can be seen in SA treated samples, with consistent expression of the housekeeping genes *Act2* and *UBQ5* in all samples (Figure 5.12).

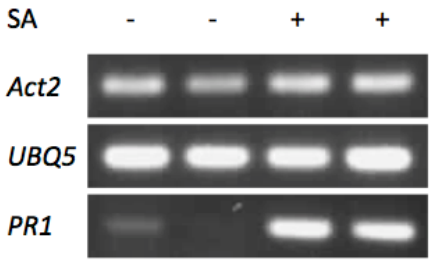


Figure 5.12: *PR1* is induced upon SA treatment.
Reverse transcriptase PCR to verify SA treatment by *PR1* induction. Two RNA samples from untreated and SA induced Col-0 seedlings were reverse transcribed and PCR was performed on RT products. PCR products for the housekeeping genes *Act2* and *UBQ5*, and the SA marker gene *PR1* are shown. Error bars display standard error, n=3.

Microarrays were then performed to compare mock and SA treated samples for *A. thaliana* Col-0, HaRxL21b and HaRxL21c. NimbleGen *Arabidopsis* 12 x 135K arrays were used, which show high reproducibility and therefore remove the need for technical replicates due to inter-array variation.

5.4.1 Data Quality

Intra and inter-array normalisation was performed within ANAIS software (Simon and Biot, 2010). To assess data quality, box plots were generated which show the overall intensity of arrays. Data is shown before (Figure 5.13a) and after (Figure 5.13b) normalisation. It can be observed that arrays 5 and 6 (HaRxL21b, untreated) showed lower overall intensity before normalisation, and that the standard deviation (after normalisation) is smaller for these arrays.

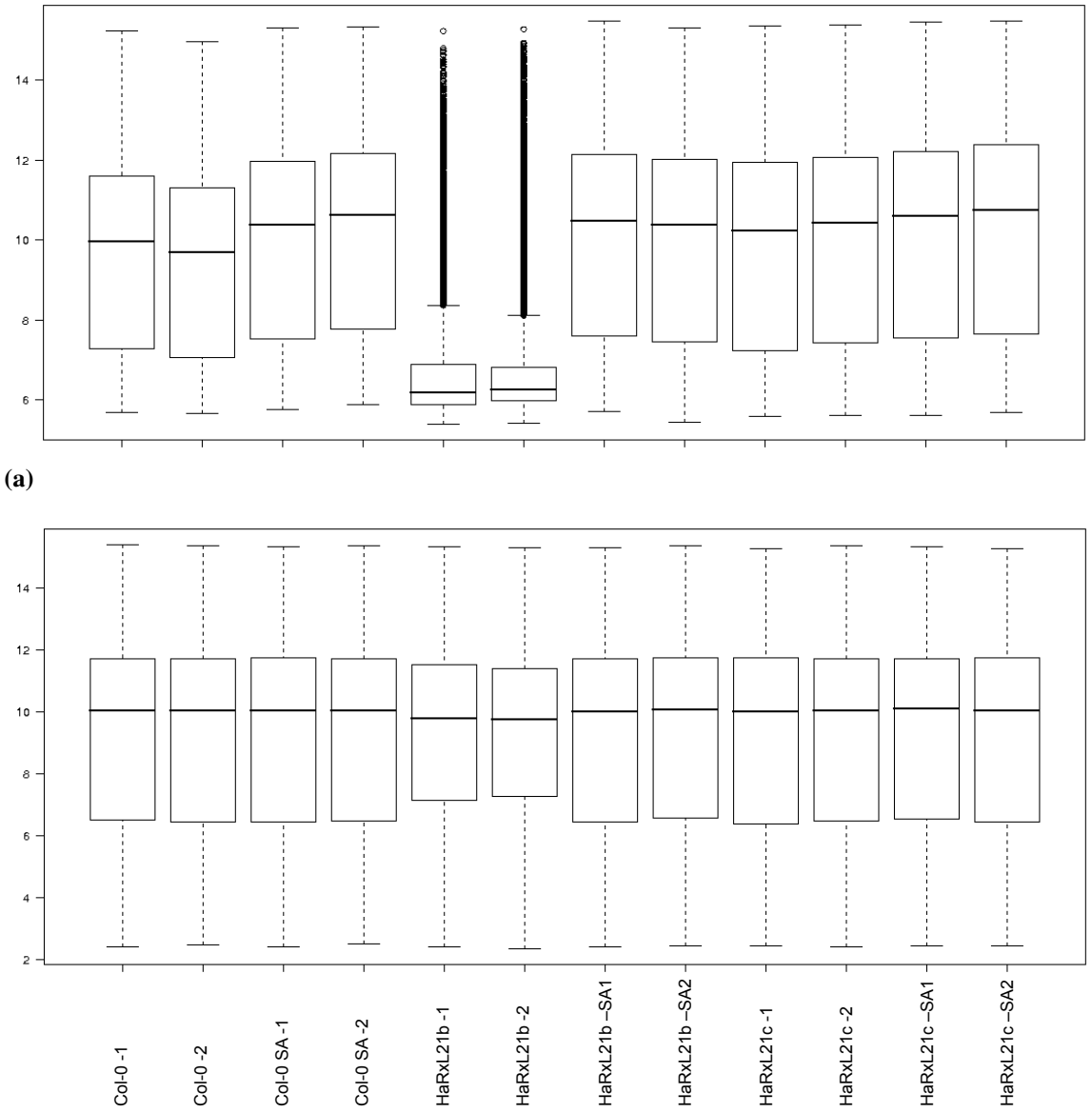


Figure 5.13: Array Intensity before and after normalisation.

Box-plots showing the overall intensities of scanned arrays (a) before and (b) after RMA and quantile normalisation. The centre is the median and box shows standard deviation, with the ends of the whiskers showing 1.5 * standard deviation. Successful normalisation is indicated by alignment of all box-plots.

5.4.2 Differential Expression before SA Treatment

To determine whether any genes were differentially expressed irrespective of SA treatment, mean gene expression across biological replicates in Col-0 was compared to mean gene expression in HaRxL21c (this was because of the poor data quality in untreated HaRxL21b).

There were 302 genes in HaRxL21c which showed greater than 2.5 fold expression compared to Col-0. Using BINGO (Maere et al., 2005), no GO-terms were found to be significantly over-represented in this group of genes. Of the 302 up regulated genes, 33 are annotated with the GO-term 'response to stress', although there is no over-representation of genes involved in ABA signalling.

There were 439 genes which showed greater than 2.5 fold down regulation in HaRxL21c compared to Col-0. No GO-terms were found to be significantly over-represented within these genes. If a cut off of greater than 5 fold reduction in gene expression is used, significant over representation of the GO-term 'response to heat' is observed due to the down regulation of the heat shock proteins AT4G25200 (ATHSP22), AT3G46230 (HSP17.4) AT1G53540 (HSP23.6) and AT4G10250 the HSP20-like chaperone protein. All of these proteins usually show up regulation in response to biotic stress, notably upon infiltration with bacterial elicitors and avirulent *P. syringae* (Winter et al., 2007).

5.4.3 Response to SA treatment

5.4.3.1 Genes induced by SA in Col-0

To ensure that SA induction was acting as expected, genes induced by SA treatment were examined. Genes were examined which were 2.5 fold up or down regulated in response to SA, using mean data of the two biological replicates. In total, 1006 genes were up regulated in response to SA and 890 genes were down regulated.

Expression data was also determined for early and late marker genes for SA response, as described by Van Den Burg and Takken (2009). Changes upon gene expression for these marker genes are shown in Table 5.3. It can be observed that there is greater induction of late SA markers, expected from this time point at 24 h post induction. Overall, the genes found to be up regulated to the greatest extent were *PR1*; showing an increase of 661 fold upon SA treatment, also *BDA1* which showed a 360 fold increase and *WRKY62*, which showed a 113 fold up regulation.

Table 5.3: Induction of SA marker genes in Col-0

NAME	ATG	FC Col-0
Early SA		
SID2	AT1G74710	1.4620
EDS1	AT3G48090	1.5028
EDS5	AT4G39030	0.6981
PAD4	AT3G52430	3.6363
NPR1	AT1G64280	1.7749
PBS3	AT5G13320	3.3273
FMO1	AT1G19250	3.7549
WRKY18	AT4G31800	1.31964
WRKY6	AT1G62300	1.2651
Late SA		
PR-1	AT2G14610	660.8761
PR-2	AT3G57260	3.5228
PR-5	AT1G75040	2.3973
NIMIN1	AT1G02450	50.4448
ATNUDT6	AT2G04450	9.4175
WRKY38	AT5G22570	79.6770
WRKY70	AT3G56400	9.7820
WRKY54	AT2G40750	19.2941
FRK1	AT2G19190	12.3897

Fold change (FC) between untreated and SA induced Col-0 of early and late SA markers (as identified by Van Den Burg and Takken (2009)).

5.4.3.2 Differential SA response due to HaRxL21

To determine whether response to SA was compromised in HaRxL21c, the fold change of late SA markers was examined between un-induced and SA treated HaRxL21c plants. Both *PR1* and *FRK1* show reduced induction upon SA treatment in HaRxL21c; 270 fold change compared to 661 fold change and 2.5 compared to 12.4 fold change respectively (Table 5.4).

Although the fold change values for HaRxL21b cannot be calculated without potentially skewing the data, the normalised gene expression levels after SA treatment (taking into account the biological replicates) show that similar gene expression levels are observed in HaRxL21b and c. For example, in the case of *PR1*, expression after SA induction in Col-0 is 19172 but in HaRxL21b and c it is 12060 and 12846 respectively (Table 5.4).

In addition to looking at marker genes, genes were identified which showed differential response to SA induction by dividing fold change upon treatment in HaRxL21c by fold change in Col-0. The result was 468 genes which showed a 3 fold or greater reduction in

Table 5.4: Induction of late SA marker genes in Col-0 and HaRxL21c

Marker Gene	FC Col-0	FC HaRxL21c	Mean Expression Col-0	Mean Expression HaRxL21c	Mean Expression HaRxL21b
PR-1	660.88	269.57	19172.69	12846.66	12060.24
PR-2	3.52	21.25	296.41	330.12	169.14
PR-5	2.40	2.12	7695.15	7974.78	6676.58
NIMIN1	50.44	49.14	8120.60	5757.08	6135.17
ATNUDT6	9.42	5.64	5106.82	3208.84	3110.94
WRKY38	79.68	147.55	2561.30	2570.52	2594.50
WRKY70	9.78	12.97	8127.70	7674.80	7691.19
WRKY54	19.29	23.27	7743.91	6515.09	5996.70
FRK1	12.39	2.51	5432.72	2499.80	2849.17

Fold change (FC) between untreated and SA induced Col-0 and HaRxL21c of late SA markers (Van Den Burg and Takken, 2009). Normalised mean expression values for SA treated plants of Col-0, HaRxL21c and HaRxL21b are also shown.

their response to SA and 620 genes which showed a 3 fold or greater response to SA in HaRxL21c compared to Col-0.

Genes which respond less to SA treatment in HaRxL21c compared to Col-0

Of the 468 genes which responded 3 fold less to SA treatment, there was found to be no significant over-representation of GO-terms when using BINGO (Maere et al., 2005). There were, however 69 genes which are annotated with the GO-term 'response to stress', many of which encoded heat shock proteins. There were also 42 genes which are annotated as having transcription factor activity (many of which were also annotated as being involved in the stress response). Examples of differentially regulated transcription factors include *WRKY8*, *53* and *71*, *NAC20* and *33*, and *MYB15*, *11*, *17*, *21*, *35*, *42*,*90*, *97* and *110*.

A hypergeometric motif test looks at motif frequency across all genes, then determines whether known transcription factor binding motifs are significantly over-represented within a gene cluster. In the 468 genes which responded 3-fold less to SA treatment in HaRxL21c compared to Col-0, 3 transcription factor binding motifs were found to be significantly over represented. The motif S-000278; ACGTOSGLUB1 ('ACGT' motif found in GluB-1 gene in rice (*Oryza sativa*)) was found to occur in 41 genes(P=3.4e-06) (Figure 5.14a). Also over-represented was the motif M01052; MYB80_01 (binding motif identified for MYB80 in wheat (Xue, 2005)) that was found to occur in 46 genes (P=0.00061) (Figure 5.14b) and motif S-000390; WBOXATNPR1 (the binding motif in the promoter of *NPR1* recognised by WRKY transcription factors (Yu et al., 2001)) which was found to occur in 35 genes (P=0.00029) (Figure 5.14c).

Genes which respond more to SA treatment in HaRxL21c compared to Col-0

Of the 620 genes which responded to a greater extent upon SA treatment, there was also found to be no significant enrichment of GO-terms. There was a relatively low number of genes annotated by 'response to stress' (60 genes) and 220 out of the 620 genes were categorised by having an 'unknown molecular function'. There were also 92 genes annotated with having 'enzyme activity', notably CYTOCHROME P450 proteins; CYP71B15, CYP71A23, CYP82C4, CYP86C3, CYP87A2, CYP96A14P, CYP702A3, CYP702A6 and CYP735A2 (also called PHYTOALEXIN DEFICIENT 3).

Performing a hypergeometric motif test identified seven transcription factor binding motifs which were over-represented in the genes which showed a greater response to SA

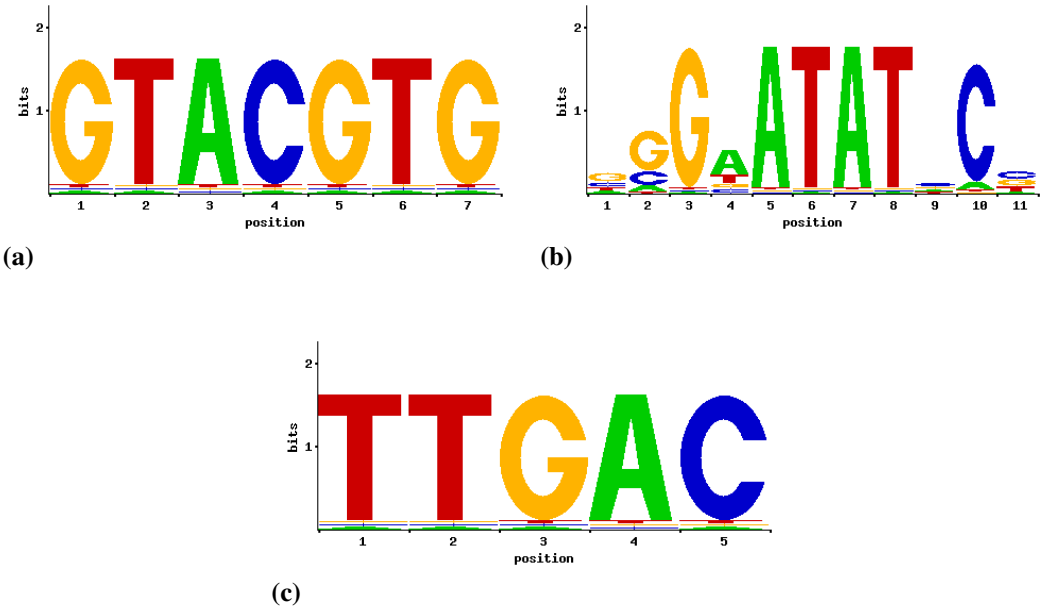


Figure 5.14: Over representation of known transcription factor binding motifs in genes which respond less to SA treatment in HaRxL21c compared to Col-0.
A hypergeometric motif test was used to identify known transcription factor binding motifs which are over represented in genes which respond less to SA treatment in HaRxL21c compared to Col-0. Three motifs were significantly over represented; (a) ACGTOSGLUB1 (P=3.4e-06), (b) MYB80_01 (P=0.00061) and (c) WBOXATNPR1 (P=0.00029).

treatment in HaRxL21c than Col-0. The motif M00089; ATHB1_01 (a motif found in the promoter of homeodomain-leucine zipper transcription factors which mediate ABA signalling (Valdés et al., 2012)) occurred in 130 of the 620 genes (P=0.00041) (Figure 5.15a). In addition, the motif M01065; ABZ1_01 (binding site for a basic leucine zipper transcription factor in tomato (Sell and Hehl, 2004)) was found in 122 genes (P=0.0004) (Figure 5.15b).

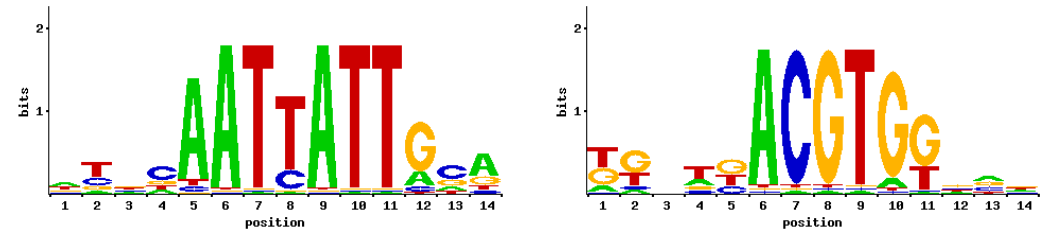
Two similar motifs which have both been implicated in the ABA response were also identified; motif S-000010; RYREPEAT4 (which has been implicated in regulation by ABA (Vasil et al., 1995)) was found in 32 genes (P=9.4e-05) (Figure 5.15c) and motif S-000102; RYREPEATVFLEB4 (bound by ABA and auxin responsive transcription factors (Hattori et al., 1992; Nag et al., 2005)) was found to occur in 37 genes (P=1.2e-06) (Figure 5.15d).

Two motifs which are thought to be involved in phytochrome A-regulated gene expression (Hudson and Quail, 2003) were also found to be over represented; motif S-000488; SORLREP3AT was found in 78 genes (P=0.00069) (Figure 5.15e) and motif S-000490; SORLREP5AT was found in 57 genes (P=0.00071) (Figure 5.15f). The first of these bears resemblance to a TATA motif and in addition, the motif S-000340; TATAPVTRNALEU ('TATA'-like motif) occurs in 98 genes (P=1.2e-05) (Figure 5.15g).

5.4.4 RT-PCR

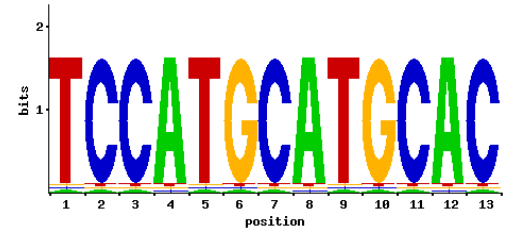
In the *A. thaliana* line HaRxL21c, reduced induction of SA marker genes upon treatment with SA was observed (Table 5.4). Microarray data from HaRxL21b was analysed with caution due to poor array hybridisation however expression levels of SA marker genes in these samples was verified using quantitative reverse transcription PCR (qRT-PCR). Relative expression of the SA marker genes *PR1* and *CBP60g* (Wang et al., 2009) in Col-0, HaRxL21b and HaRxL21c was determined through normalisation relative to the housekeeping genes *UBQ5* and *TUB6*, qRT-PCR was performed by Jens Steinbrenner.

For both *PR1* and *CBP60g*, induction upon SA treatment was reduced in both HaRxL21b and HaRxL21c compared to Col-0. (Figure 5.16a and Figure 5.16b)



(a)

(b)



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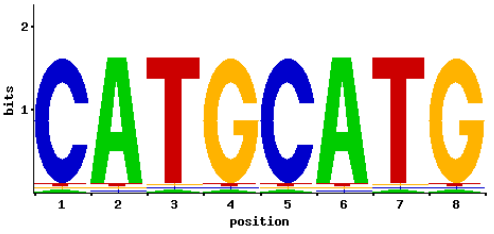
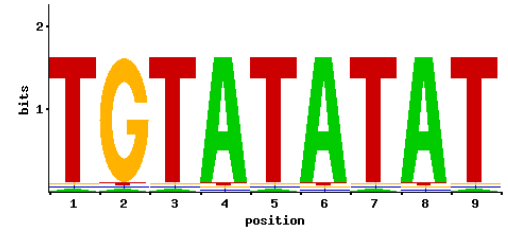
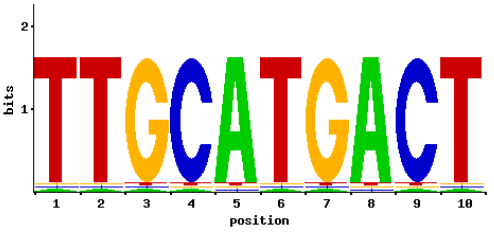


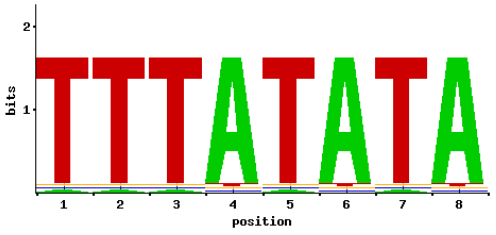
(d)



(e)

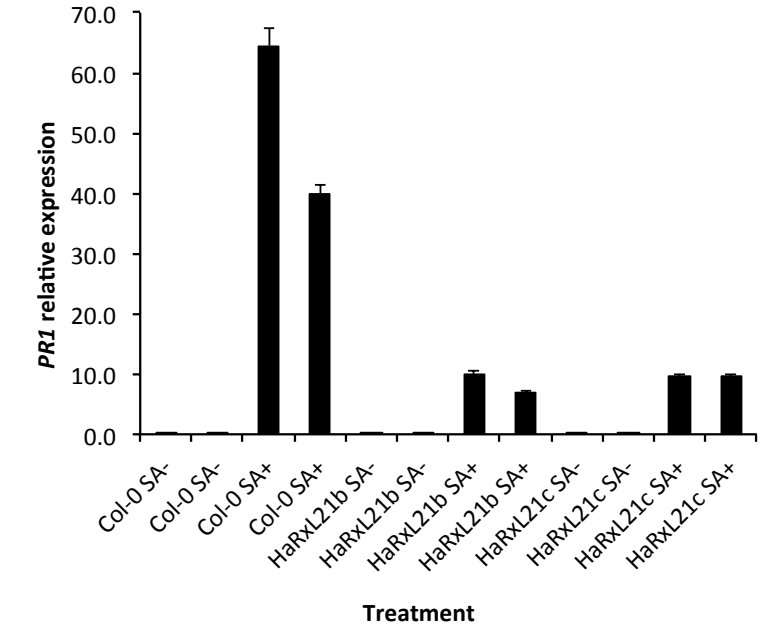


(f)

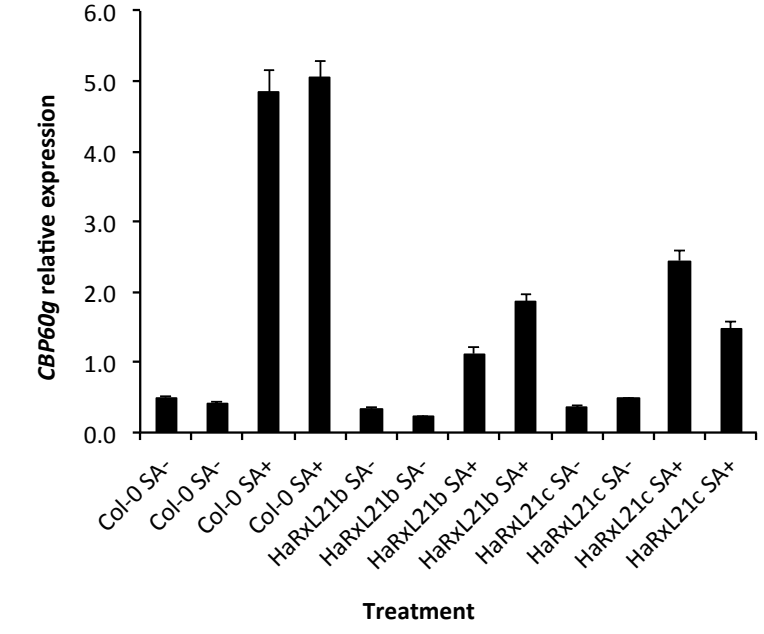


(g)

Figure 5.15: Over representation of known transcription factor binding motifs in genes which respond more to SA treatment in HaRxL21c compared to Col-0.
A hypergeometric motif test was used to identify known transcription factor binding motifs which are over represented in genes which respond more to SA treatment in HaRxL21c compared to Col-0. Seven motifs were significantly over represented; (a) ATHB1_01 (P=0.00041), (b) ABZ1_01 (P=0.0004), (c) RYREPEAT4 (P=9.4e-05), (d) RYREPEATVFLEB4 (P=1.2e-06), (e) TATAPVTRNALEU (P=1.2e-05), (f) SORLREP3AT (P=0.00069) and (g) SORLREP5AT (P=0.00071). P-values represent significant up regulation in this gene cluster.



(a)



(b)

Figure 5.16: HaRxL21 suppresses induction of the SA marker genes *PR1* and *CBP60g*.

HaRxL21 expression *in planta* suppresses expression of (a) *PR1* and (b) *CBP60g* after treatment with SA dissolved in DMSO (SA+). Expression due to treatment with DMSO alone (SA-) is also shown. Expression was determined by quantitative reverse transcription PCR (qRT-PCR) and normalised to *UBQ5* and *TUB6* expression. Two biological replicates are shown for each treatment, error bars show standard error between technical replicates (n=3). qRT-PCR was performed by Jens Steinbrenner.

5.5 Discussion

5.5.1 *B. cinerea* microarrays

Differences Prior to Infection

Microarrays were performed on HaRxL21a and b, and compared to wild type before and during *B. cinerea* infection. Initial observations concern the differentially expressed genes in uninfected tissue which are likely to give an indication of how the effector is altering transcriptional processes simply through its presence within the plant.

Looking at genes which are significantly differentially expressed in both HaRxL21a and HaRxL21b compared to Col-0 gives a set of 138 genes which can be treated as 'high confidence'. Within this set, genes categorised by GO-terms indicating response to stress are more prominent in the 65 up-regulated genes, for example 'response to hormone stimulus', specifically ABA. When the mean gene expression of HaRxL21a and HaRxL21b is considered, a larger set of differentially expressed genes is gained because genes which are slightly outside the threshold for significance in either line are included. Again, genes involved in ABA signalling were found to be up regulated and many other genes involved in stress responses and response to pathogens were also found to be differentially regulated. It is also interesting to note that H2A proteins were found in both up and down regulated gene sets.

A possible mechanism for HaRxL21 activity in the host is suggested by these data. Interaction between signalling pathways mediated by plant hormones is complex and in many cases antagonistic. It is known that ABA suppresses SA mediated signalling (Audenaert et al., 2002) and JA / ET signalling (Anderson et al., 2004). Manipulation of hormone antagonism (in particular ABA signalling) is a virulence strategy employed by the phytopathogen *P. syringae*; it has been shown that ABA accumulates in response to *P. syringae* pv. *tomato* DC3000 but not in response to a *hrpA* strain (which is deficient in type III secretion) therefore showing that type III effectors are responsible for this effect (de Torres-Zabala et al., 2007, 2009). In addition, pre-treatment with ABA has been shown to enhance susceptibility to oomycete pathogens (Ward et al., 1989; McDonald and Cahill, 1999; Mohr and Cahill, 2003). These data suggest that that if HaRxL21 is able to enhance levels of ABA or sensitivity to ABA, signalling mediated by SA and JA could be antagonised and inhibited. This hypothesis is supported by the data; both the SA marker gene *PR1* and the JA marker genes *PDF1.2* and *MBP2* are shown to be down regulated.

A cautionary note however, is that some of the genes which are differentially expressed

prior to infection, such as MBP1 and 2 are usually only expressed at very low levels in uninfected tissue (Winter et al., 2007). It is possible that some level of stress was exerted on the plants since this was a detached leaf assay, therefore what we observe here is the plant lines responding differently, resulting in differences which are observed in the uninfected tissue. It is also possible however that genes may be significantly differentially expressed but because the actual expression values are so low it is of little biological significance.

It was also observed that there were many genes which were differentially regulated between HaRxL21a and HaRxL21b. These are two independently transformed *A. thaliana* lines expressing 35S::HaRxL21, and it can be assumed that HaRxL21 is inserted in different locations in the *A. thaliana* genome. HaRxL21a has been observed to display a small phenotype (Figure 3.1) which is not observed in HaRxL21b or c. It is therefore possible that there are many transcriptional differences between HaRxL21a and b due to these developmental differences. If these arrays were repeated, HaRxL21b and c would be chosen as these lines display a phenotype more close to Col-0, while still exhibiting enhanced susceptibility. However it was interesting to observe that despite obvious phenotypic differences between HaRxL21a and Col-0, it displayed less differentially expressed genes with Col-0 than HaRxL21b, perhaps indicating that transcriptional differences leading to the small phenotype occurred at earlier developmental stages. Because the effector here is expressed *in planta* without any tag on the protein, it is not possible to detect protein expression level without a specific antibody to the effector. However RNA levels show that *HaRxL21* is expressed at slightly lower levels in HaRxL21a compared to HaRxL21b (Figure 3.2), which may result in fewer genetic differences from the Col-0 plants. This may also contributes to the lack of increased susceptibility of HaRxL21a to *Hpa*, previously thought to be due to the small growth phenotype.

Differences Post Infection

Rowe et al. (2010) identified genes and pathways which had altered transcription levels in response to *B. cinerea* infection. Pathways which showed enhanced transcription were found to be camalexin biosynthesis, tryptophan biosynthesis, glutathione transferases, lignin biosynthesis, jasmonate biosynthesis and response and senescence-associated genes. In total, they found that found that 11,989 of 22810 transcripts on their arrays showed significant changes with *B. cinerea* treatment. In addition, Windram et al. (2012) have generated a high resolution time course of *B. cinerea* infection. The data here shows concordance with both of these data sets, with a large number of genetic changes occurring

within 22 hours of infection.

The microarray data showed very few differentially expressed genes post-infection with *B. cinerea* between Col-0 and HaRxL21-expressing lines. It is possible that this is due to transcriptional changes brought about by *B. cinerea*, which may have some overlap with the effects of HaRxL21 by the action of it's own effector proteins. It is also possible that by 22 hours post infection, the infection was had progressed to an extent that transcriptional changes in response to infection were masking any effects caused by HaRxL21. Another explanation is that changes in gene expression brought about by HaRxL21 expression *in planta* 'primed' the plant for susceptibility, therefore causing *B. cinerea* to colonise the leaf more rapidly. This may be visible using a time series, as a forward 'shift' in time of transcriptional changes due to infection may be observed. This hypothesis could be validated by qPCR at smaller time intervals post infection, establishing whether there is a time-shift forwards of gene-expression changes both mediated by *B. cinerea* and by the plant in response to the infection.

When identifying genes which respond differently to infection, it is important to take into account significantly different responses but also the magnitude of change, for example a gene which responds a tiny amount to infection and a tiny amount more in another plant line may not have any biological relevance. To aid in deciphering the action of HaRxL21, data obtained here can be compared to published high resolution time series data. The four genes found to be usually up regulated upon *B. cinerea* infection but less in the presence of HaRxL21 have been categorised into gene expression clusters by Windram et al. (2012). AT1G77450 (ANAC032) is in cluster 36 (Windram et al., 2012) and expected to show peak expression at around 24h post infection. Because only one time point is considered in the data here, we cannot see whether this expression profile has been shifted in time or whether the gene is simply being expressed less in HaRxL21a and b. AT1G02830 (AtGAT1) is in cluster 1 which starts to be down regulated at around 24 h post infection with *B. cinerea*, suggesting that the response to infection may be sped up by the presence of HaRxL21. On the other hand, AT1G02850 (BGLU11) is in cluster 42 and AT1G62570 (FMO_GS-OX4) falls into cluster 30, both of which show an increase in gene expression until around 28 hours post infection.

5.5.2 Hormone assays

JA Root Length Inhibition

Root growth inhibition by JA can be used as an indicator of disruption to JA signalling, for example in the case of the co-repressor NINJA which negatively regulated JA signalling and therefore when over expressed suppresses root growth inhibition (Pauwels et al., 2010).

Results here that *in planta* expression of HaRxL21 suppressed JA-mediated inhibition of root growth may indicate that HaRxL21 expression causes suppression of JA-responsive genes. This is in agreement with microarray data which suggested a down regulation of marker genes for JA signalling such as PDF1.2. These data are also in concordance with the model that enhanced ABA signalling in HaRxL21 plants results in antagonism of JA signalling. However, it can be observed that in this experiment HaRxL21b did not show a significant reduction of root growth inhibition in the presence of MeJA. HaRxL21b exhibited the strongest susceptibility phenotype to both the *Hpa* isolate Noks1 and *B. cinerea* (Figures 3.5a and 3.8) so this result should be considered with caution as it may not be representative of what HaRxL21 is doing in the plant.

Different root lengths in the absence of JA (it was generally observed that 35S::HaRxL21 lines had shorter roots) may be indicative of altered hormone balances in the plants. For example enhanced ABA and JA levels may inhibit root growth (Pilet, 1975).

ABA Germination

The results here suggested that HaRxL21 expression *in planta* showed an increased sensitivity to ABA, showed by the significantly increased germination inhibition in the presence of 0.5 mM ABA in HaRxL21b, and a reduction in germination rate which can also be observed in HaRxL21c. This observation would be concurrent with observations in the microarray data which showed an up regulation of ABA signalling pathways in uninfected leaf tissue (Figure 5.6). Difficulty in repeating these results has occurred due to varying rates of germination amongst Col-0 seeds. Seed germination is influenced by the phytohormones gibberelic acid (GA) and ABA (Debeaujon and Koornneef, 2000), however it has also been shown that both the season in which seeds are harvested and seed storage conditions have an impact on seed dormancy (Derkx and Karssen, 1993). This may explain why reproducibility of germination experiments using HaRxL21a-c has been hindered by varying germination rates amongst Col-0 seeds.

Transgenic lines expressing HA::HaRxL21 and HA::GFP have now been generated, which were harvested at the same time and have been stored under the same conditions. Therefore resources are now available in which future germination experiments could be performed without the season of harvest and seed storage conditions altering germination rates between plant lines. It has been observed that these lines show poor germination and that this effect is stronger when higher levels of HaRxL21 are present, although this effect is yet to be quantified. These lines may give additional insights into the role of ABA and whether the EAR motif-mediated interaction with TPL is required for this effect.

Protoplasts

The results obtained using *A. thaliana* mesophyll protoplasts showed that HaRxL21 inhibited *PR1* induction, 6 hours after treatment with SA, compared to GFP as a control. This result was not observed when the EAR motif was either deleted or when the Leucine residues were mutated to Phe, indicating that the interaction of HaRxL21 with TPL is important for this effect. To verify that HaRxL21 suppression of *PR1* activation is mediated by interaction with TPL, Col-0 PR1::GUS plants are being crossed with Col-0 *tpr1-tpl-tpr4* plants to see whether the difference between HaRxL21 and GFP is removed in the absence of TPL and its close homologues.

SA signalling mediates defense against biotrophic pathogens, characterised by expression of the marker gene *PR1* which is suppressed by HaRxL21. Therefore a hypothesis as to why *A. thaliana* plants expressing HaRxL21 show enhanced susceptibility to *Hpa* is that by interfering with SA signalling in this way, this effector enhances susceptibility to *Hpa*.

This assay assumes that all plasmids are taken up equally, and therefore adjusts GUS measurements based on luciferase activity. Any differences in transfection between luciferase and the effector may contribute to values being skewed, however the same result was obtained by multiple independent experiments suggesting that this system is robust. SA induction and subsequent gene expression *in planta* (rather than in the protoplast system which is somewhat artificial) can be used to validate this data.

5.5.3 SA microrrays

During assessment of data quality, it was shown that two arrays (arrays 5 and 6, HaRxL21b untreated replicates 1 and 2) were less intense. This may be because the cDNA labelling didn't work as well, although the same total amounts of labelled cDNA were loaded onto

each array. Normalisation has transformed the raw data from these arrays, however the implications of this are that the data for these arrays may be more exaggerated; genes which are slightly up or down regulated will show increased up or down regulation. It is therefore important to validate transcriptional effects observed in HaRxL21 by looking at results from the independently transformed line HaRxL21c. However it must be considered that HaRxL21b has often showed the most susceptible plant phenotype (Figures 3.5a and 3.8) so some pronounced gene expression differences to Col-0 may be real.

Differences prior to infection were examined using HaRxL21c alone due to lack of confidence in data for un treated HaRxL21b. The differentially expressed genes included heat shock proteins, which have been shown to be involved in response to stress, including signalling molecules in plant hormone pathways (Wang et al., 2004). However the up regulation of ABA signalling previously observed in HaRxL21a and b prior to *B. cinerea* infection was not observed. It is important to note that these microarrays were performed using different plant growth conditions to *B. cinerea* arrays. Even the uninfected tissue will be different as plants were different ages and grown under different conditions (soil for *B. cinerea* infection vs MS agar plates prior to SA treatment which may cause more stress). Also *B. cinerea* infection was performed using a detached leaf assay so some early wounding responses may have triggered transcriptional responses before the 0 h time point was harvested.

Differentially expressed genes in Col-0 upon treatment show that SA induction worked; the up-regulation of genes such as *PR1* and other SA markers can be observed. Interestingly upon infection, *PR1* activation appears to be reduced in HaRxL21c but not *PR2*, suggesting that the action of HaRxL21 is specific. Although changes upon infection cannot be calculated for HaRxL21b, *PR1* expression levels post SA induction are similar in HaRxL21b and c, therefore suggesting that observations are reproducible. This suppression of *PR1 in planta* is in concordance with observations in protoplasts.

During SA induction, in addition to suppression of *PR1* activation there were many genes which responded 3-fold more or less in HaRxL21c compared to Col-0. In the subset of genes which responded less, the 'W-Box NPR1' motif (TTGAC) was found to be over-represented. This motif occurs multiple times in the promoter of NPR1 (Figure 5.17) and has been linked to binding by WRKY transcription factors; mutations in this motif prevent binding by WRKY18 (Yu et al., 2001). It is therefore possible that HaRxL21 is achieving suppression of SA responsive genes through binding (either directly or indirectly) to a WRKY transcription factor.

Genes which responded to a greater extent upon SA induction in HaRxL21-expressing plants showed an over representation of transcription factor binding motifs involved in

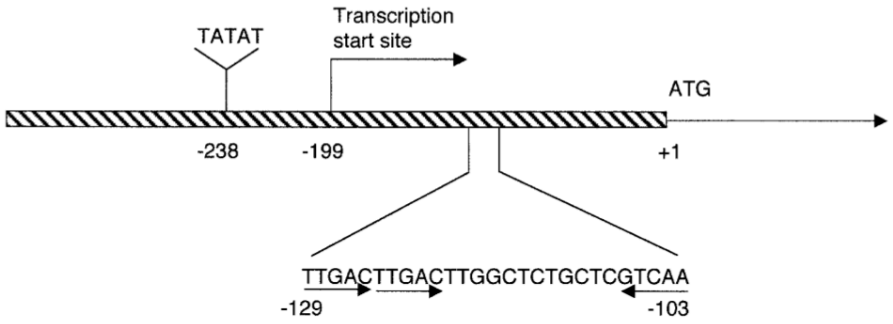


Figure 5.17: The Arabidopsis *NPR1* promoter.
Schematic of the Arabidopsis *NPR1* promoter, showing the TTGAC motif. Reproduced with permission from (Yu et al., 2001).

ABA signalling. This may suggest concurrence with the results from the microarrays performed during *B. cinerea* infection; that ABA signalling was enhanced. Measurements for hormone levels in these plants would aid in deciphering whether HaRxL21 is targeting ABA responsive promoters or whether levels of ABA are higher.

Limitations of microarray data must also be considered, for example Pérez-Amador et al. (2001) found that 90% of genes which showed a greater than 1.5-fold difference in a single experiment were not reproducibly different in subsequent analyses. Therefore results need to be verified via other means. Quantitative RT-PCR to check for induction levels of the SA marker genes *PR1* and *CBP60g* showed lesser induction in *A. thaliana* plants expressing 35S::HaRxL21 (Figure 5.16), giving confidence that effects observed were not an artefact of arrays. Future work to confim whether the EAR motif in HaRxL21 is necessary for suppression of SA signalling can be done using HA::HaRxL21 plants and HA::HaRxL21ΔEAR plants which have recently been generated. These plants are expressing the same constructs which were used to transfect the mesophyll protoplasts and therefore it is expected that results *in planta* should reflect results obtained in the protoplast system (although expression levels may impact results).

5.5.4 Summary

- In detached but uninfected leaves, the expression of HaRxL21 in *A. thaliana* causes up regulation of genes involved in ABA signalling compared to Col-0, and down regulation of marker genes for SA (*PR1*) and JA (*MBP2* and *PDF1.2*) signalling.
- HaRxL21 suppresses induction of the SA signalling marker gene *PR1* both when transiently expressed in *A. thaliana* protoplasts and when stably transformed into *A. thaliana*. *PR1* induction in *A. thaliana* protoplasts transfected with HaRxL21ΔEAR

is not suppressed.

- Genes which respond to SA treatment to a lesser extent in HaRxL21-expression *A. thaliana* plants show a significant over-representation of the transcription factor binding motif for MYB80 and the WRKY transcription factor-associated 'W BOX' found in the promoter region of *NPR1*.

Chapter 6

Potential mechanisms for HaRxL21 action

6.1 Introduction

In additional to large scale phenotypic screens for virulence contributions of effectors (such as Fabro et al. (2011)), the molecular mechanism by which pathogen effectors perform their function is beginning to be elucidated. Examples include the *P. syringae* effectors AvrPphB (which cleaves the *A. thaliana* protein kinase PBS1 (Shao et al., 2003)) and HopZ1a (which interacts with and acetylates tubulin and through disruption of the microtubule network, disrupts plant defense (Lee et al., 2012)). The *Pi* effector AVR3a (which is essential for *Pi* virulence) has been found to act by interacting with and stablise the E3 ligase CMPG1, therefore interfering with programmed cell death (Bos et al., 2010).

The aim here is therefore to begin to unravel the biochemical mechanism by which the *Hpa* effector HaRxL21 induces changes in host transcription and therefore enhances virulence of *Hpa*.

6.1.1 The 26S proteasome

Protein turnover is vital to cells as damaged or misfolded proteins must be degraded, in addition being key to many cellular processes including cell cycle progression and response to stress (King et al., 1996; Worley et al., 2000; Thines et al., 2007). Prior to discovery of the 26S proteasome it was assumed that the site of protein degradation was the lysosome due to the high concentrations of proteases present in this organelle and

its involvement in degrading endocytosed proteins. However an ATP-dependent protein degradation system in addition to lysosomes was found to be responsible for degrading abnormal proteins in reticulocytes (immature red blood cells) (Etlinger and Goldberg, 1977). It was also shown that this system showed selectivity and depends on proteins being marked for degradation by ubiquitin; a highly conserved molecule in eukaryotes (Callis and Vierstra, 1989; Wilkinson et al., 1980; Hershko et al., 1983).

The *Hpa* effector HaRxL21 interacts with the host (*A. thaliana*) protein TPL in the nucleus (Figure 4.18). During Co-Immunoprecipitation of HaRxL21 with TPL, it was also observed that TPL is detected to a higher level in the presence of HaRxL21, an effect which is not observed when TPLΔCTLH is used or when full length TPL is expressed in the presence of HaRxL21ΔEAR (thus preventing the interaction from occurring) (Figure 4.20). It is therefore hypothesised that HaRxL21 may be somehow stabilising TPL upon interaction.

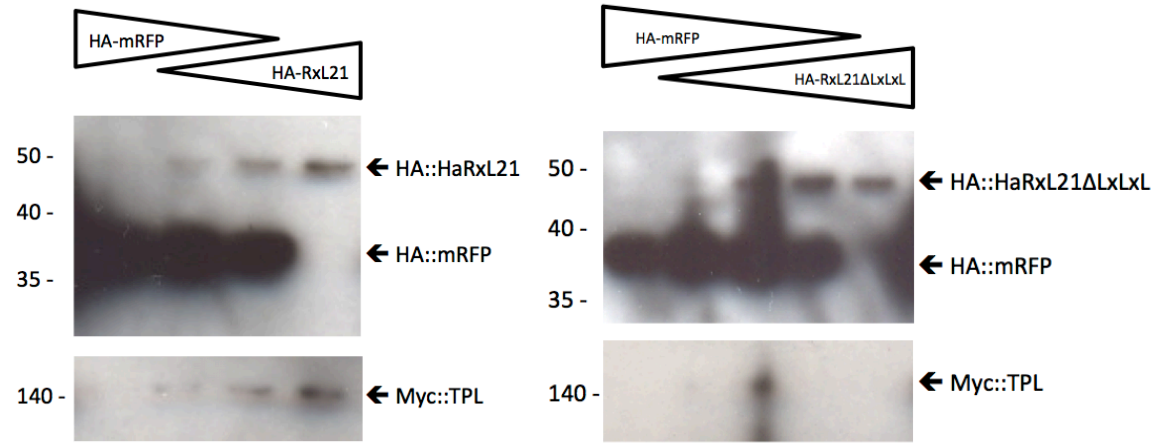
Genes which encode components of the ubiquitin/26S proteasome pathway have been estimated to exceed 1300, with this complexity highlighting the importance of its regulatory ability (Vierstra, 2003). For example, a role for the 26S proteasome has been shown in the regulation of the JA response through the selective recruitment of TPL which is relieved upon degradation of the JAZ repressor proteins (Thines et al., 2007; Pauwels et al., 2010). The similarity of this system to auxin signalling which depends on degradation of Aux/IAAs has been noted (Worley et al., 2000; Pérez and Goossens, 2013). Turnover of TPL itself (as opposed to degradation of co-repressors) has not been previously described. To study turnover by the proteasome, tools such as MG132 (carbobenzoxy-Leu-Leu-leucinal) which inhibits the proteolytic activity of the 26S proteasome are available (Lee and Goldberg, 1998). MG132 can therefore can be used to establish whether TPL is turned over in the cell and HaRxL21 interferes with this process through protein-protein interaction.

6.2 TPL Stabilisation

To test whether the presence of HaRxL21 stabilises TPL, *N. benthamiana* was transiently co-transformed with *A. tumefaciens* harbouring constructs encoding Myc::TPL and HA::HaRxL21 (using the method described in section 2.2.5.3). The amount of Myc::TPL was kept constant, and leaves were infiltrated with increasing amounts of *A. tumefaciens* harbouring the construct encoding HaRxL21, with the consequence that more protein was produced. The rationale behind this is that if HaRxL21 does stabilise TPL then increasing amounts of TPL should be detected with increasing amounts of HaRxL21.

The amount of protein expressed can be controlled by adjusting the optical density of *A. tumefaciens* infiltrated. To determine whether this stabilisation effect was specific to HaRxL21 (as opposed to an artefact caused by protein over-expression), the same optical density of *A. tumefaciens* was used for each infiltration. Lower amounts of *A. tumefaciens* encoding HA::HaRxL21 were buffered with *A. tumefaciens* encoding HA::mRFP, resulting in a gradient of relative amounts of HA::HaRxL21 and HA::mRFP. The amount of *A. tumefaciens* encoding Myc::TPL was kept constant (see method 2.2.11.4). To detect protein levels, western blots were performed (method described in section 2.2.11.2) and membranes were probed with α-HA and α-Myc.

It was observed that when the ratio of HA::HaRxL21 to HA::mRFP was increased, a corresponding increase in TPL was observed (Figure 6.1a). This effect was not observed when HA::HaRxL21ΔEAR was co-expressed with TPL (Figure 6.1b).



(a) **(b)**

Figure 6.1: HA::HaRxL21 stablises TPL in *N. benthamiana* but HA::HaRxL21ΔEAR does not.

Western blot showing that HaRxL21 stablises TPL. (a) HA::HaRxL21 and (b) HA::HaRxL21ΔEAR were co-expressed with Myc::TPL by *A. tumefaciens* mediated transient transformation of *N. benthamiana*. The total OD_{600} of *A. tumefaciens* infiltrated was kept constant and changing amounts of *A. tumefaciens* harbouring a construct encoding HA::HaRxL21 or HA::HaRxL21ΔEAR amounts were buffered with *A. tumefaciens* encoding HA::mRFP. *A. tumefaciens* harbouring a construct encoding Myc::TPL was infiltrated at at the same OD_{600} in all samples.

6.3 TPL Turnover

Turnover of TPL by the 26S proteasome has previously not been previously described. To establish whether this increase in stability in the presence of HaRxL21 (Figure 6.1a) was due to prevention of TPL degradation by the proteasome, the effect of MG132 was examined. MG132 is an inhibitor of the ubiquitin proteasome (Lee and Goldberg, 1998), therefore protein which is usually turned over by the proteasome should accumulate in the presence of this inhibitor.

HA::TPL and HA::TPLΔCTLH were produced *in planta* using *A. tumefaciens* mediated transient transformation of *N. benthamiana*, using co-infiltration with the P19 silencing suppressor to enhance expression (Voinnet et al., 2003) as described in section 2.2.5.3. Leaves were subsequently treated by infiltration with either 100 μM MG132 in DMSO or DMSO alone. Total protein level in each sample was equalised using Bradford Ultra reagent (Expedeon) and TPL / TPLΔCTLH levels detected by western blot against α-HA. It can be observed that protein detection is higher in samples which received MG132 treatment (Figure 6.2). It can also be observed that HA::TPLΔCTLH is present at lower levels than full length HA::TPL in MG132 treated samples and cannot be detected in samples which have not been treated with MG132. Data is shown for two samples from independently transformed leaves for each treatment and time point.

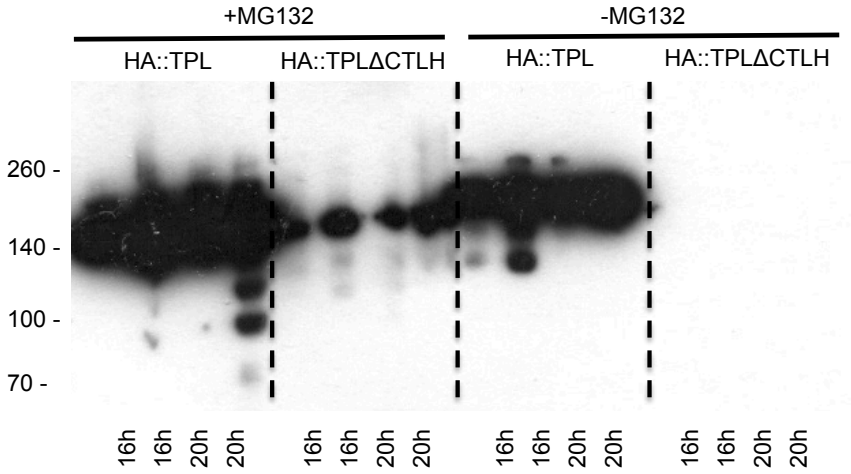


Figure 6.2: TPL shows reduced stability in the absence of the CTLH domain and is turned over by the 26S proteasome.
HA::TPL and HA::TPLΔCTLH were expressed by *A. tumefaciens* mediated transient transformation of *N. benthamiana*. Forty eight hours post infiltration, leaves were treated with 100 μM MG132 in DMSproteasomeO (+MG132) or DMSO alone (-MG132) and harvested 16 and 20 hours post treatment. Each treatment was performed in two independently transformed leaves. Gel loading was adjusted so total protein levels were equal and a western blot was performed to detect protein using α-HA.

6.4 Discussion

6.4.1 TPL Stability and Turnover

An increase in stability of TPL is observed in the presence of HaRxL21 in a dose-dependent manner; higher amounts of HaRxL21 result in higher amounts of TPL accumulation. It has also been observed that TPLΔCTLH shows a lower stability compared to full length TPL, and that stability of both TPL and TPLΔCTLH is increased after treatment with the proteasome inhibitor MG132. TPL from *N. benthamiana* is similar to TPL in *A. thaliana* and the CTLH domain is conserved between the two plant species (Figure 4.11). Therefore it is likely that we see this effect even using transient expression in *N. benthamiana* because of the conserved mode of action of TPL / TPR proteins across plant species (as previously described by Causier et al. (2012)). These data suggest that TPL is degraded in the absence of an EAR-motif interaction through turnover by the 26S proteasome and that interactions between EAR repressors and the CTLH domain of TPL bring about an 'active' state which is no longer degraded. This is likely to be brought about by either conformational change due to protein binding or by post translational modification. Further work needs to be carried out to establish the mechanism by which HaRxL21 stablises TPL. Using another nuclear localised effector as an additional control could ensure that this observation was not simply a over-expression effect which does not occur when using mRFP.

It has been postulated that another level of regulation of TPL / EAR-motif mediated repression may be brought about by post-translational modification (Kagale and Rozwadowski, 2011). The Groucho (Gro) / TLE family of proteins (in *Drosophila* and mammals respectively) show structural similarity to TPL and TPRs (Liu and Karmarkar, 2008), specifically the presence of WD-repeat motifs (consisting of tryptophan (W) and aspartate (D) residues). These proteins have been extensively studied and like TPL, play a role in transcriptional repression in a variety of different pathways through the recruitment of histone deacetylases (Chen and Courey, 2000), they may therefore provide clues as to how transcriptional repression brought about by TPL is regulated. The importance of post-translational modification of Gro has been shown; both phosphorylation (Nuthall et al., 2002, 2004) and SUMOylation (Ahn et al., 2009) have been shown to be necessary for full co-repressor activity. Subsequently, post-translational modification of TPL has been indicated using mass spectrometry, revealing phosphorylation of Tyr133, Ser214 and Thr286 (Heazlewood et al., 2008; Durek et al., 2010). In addition, 2 protein kinases and a phosphatase have been identified as interacting partners of TPL, with a potential role in post-translational modification of TPL (Kagale S and Rozwadowski K, unpublished).

These data and observations here (Figure6.2) may indicate that interacting partners of TPL bring about post-translational modification which prevent degradation by the 26S proteasome and enable transcriptional repression through the recruitment of HDA19.

Investigation of post-translational modification of TPL upon interaction with HaRxL21 could be performed using phosphatase inhibitors such as λ-phosphatase, which removes phosphate groups from phosphorylated serine, threonine and tyrosine residues. The result may be a shift in TPL mobility which could be observed on a western blot. Antibodies against post-translational modifications could also be used, for example phospho-specific antibodies or antibodies against SUMO-1,2 and 3 to indicate whether SUMOylation is taking place. Low expression levels in TPLΔCTLH may cause investigation of post translational modification to be difficult, however. An alternative vector system which could be used to enhance expression of TPL is described by Kagale et al. (2012). This system uses tobacco mosaic virus based vectors which can produce higher (100 fold more) recombinant protein than using a P19 enhanced system (Voinnet et al., 2003) as used previously here. Using this system may enable higher expression of TPL and in particular TPLΔCTLH, therefore enabling investigation of potential differences in post translational modification when the CTLH domain is deleted and also in the presence of HaRxL21.

6.4.2 Possible Model for HaRxL21 function

Using the data here and available literature, the following model for the action of TPL and how this is manipulated by HaRxL21 is proposed.

In it's 'inactive' state (purple in Figure 6.3) when nothing is interacting with the CTLH domain, TPL is turned over by the 26S proteasome (Figure 6.3a). When expressed without the CTLH domain (therefore preventing interactions with EAR-motif containing proteins from occurring) reduced protein stability is observed (Figure 6.2). It should be noted that this state is likely to be rare in plants due to the large number of protein-protein interactions which have been detected for TPL and TPRs (Causier et al., 2012). It is also plausible that in this state, TPL does not interact with HDA19, although this has not been investigated.

TPL functions as a general repressor, with specificity mediated by the complex which forms between the CTLH domain of TPL and the transcription factor target for repression (indicated in Figure 6.3b). Examples in the literature include NINJA which recruits TPL to jasmonate signalling (Pauwels et al., 2010), NIMIN which links TPL to SA signalling (Weigel et al., 2005; Arabidopsis Interactome Mapping Consortium, 2011), IAAs which link TPL to auxin signalling (Long et al., 2006), TIE1 which links TPL to TCP tran-

scription factors (Tao et al., 2013) and APETALA2 which recruits TPL to regulate floral organ identity (Krogan et al., 2012). Recruitment of TPL by proteins which are able to directly bind DNA has been described Wang et al. (2013). Repression of transcription is subsequently mediated by TPL entering an 'active' state (blue in Figure 6.3) which is not turned over by the 26S proteasome. The histone deacytelase HDA19 is recruited, resulting in acetylation of histones and therefore transcriptional repression. It is worth noting that although this protein has been shown to interact with TPL (Krogan et al., 2012), direct interaction has not been proven and this interaction may be mediated by other proteins such as SAP18 (Zhang et al., 1997; Song and Galbraith, 2006).

Removal of repression is mediated by 'stress receptors' which are targeted for degradation by the 26S proteasome in response to stress. For example JAZ proteins which degrade in the presence of JA-Ile (Thines et al., 2007; Chini et al., 2007), Aux/IAAs which degrade in response to Auxin (Worley et al., 2000) and NPR1 which degrades in the presence of SA (Fu et al., 2012); the similarity of the mechanims which mediate SA and auxin signalling has been noted (Pérez and Goossens, 2013). The result of the degradation of these proteins is that TPL is no longer bound by protein-protein interactions to the transcription initiation site and therefore histone deacetylation is relaxed, allowing transcription (Figure 6.3c). The mechanism by which histone acetylation occurs is currently unknown, although it is possible that acetylated histones are the 'resting state' given the active recruitment of histone deacetylases by the TPL / EAR repressor complex.

HaRxL21 has been shown to physically interact with TPL and cause its stabilisation (Figure 6.1a), possibly due to post translational modification which causes the 'active' form of TPL to be recruited to a transcription initiation site (Figure 6.3d). Possible targets include WRKY transcription factors as indicated by the over-representation of 'W Box NPR1' in microarray data. It is also possible that HaRxL21 directly binds to target DNA however no evidence for this has been found so far.

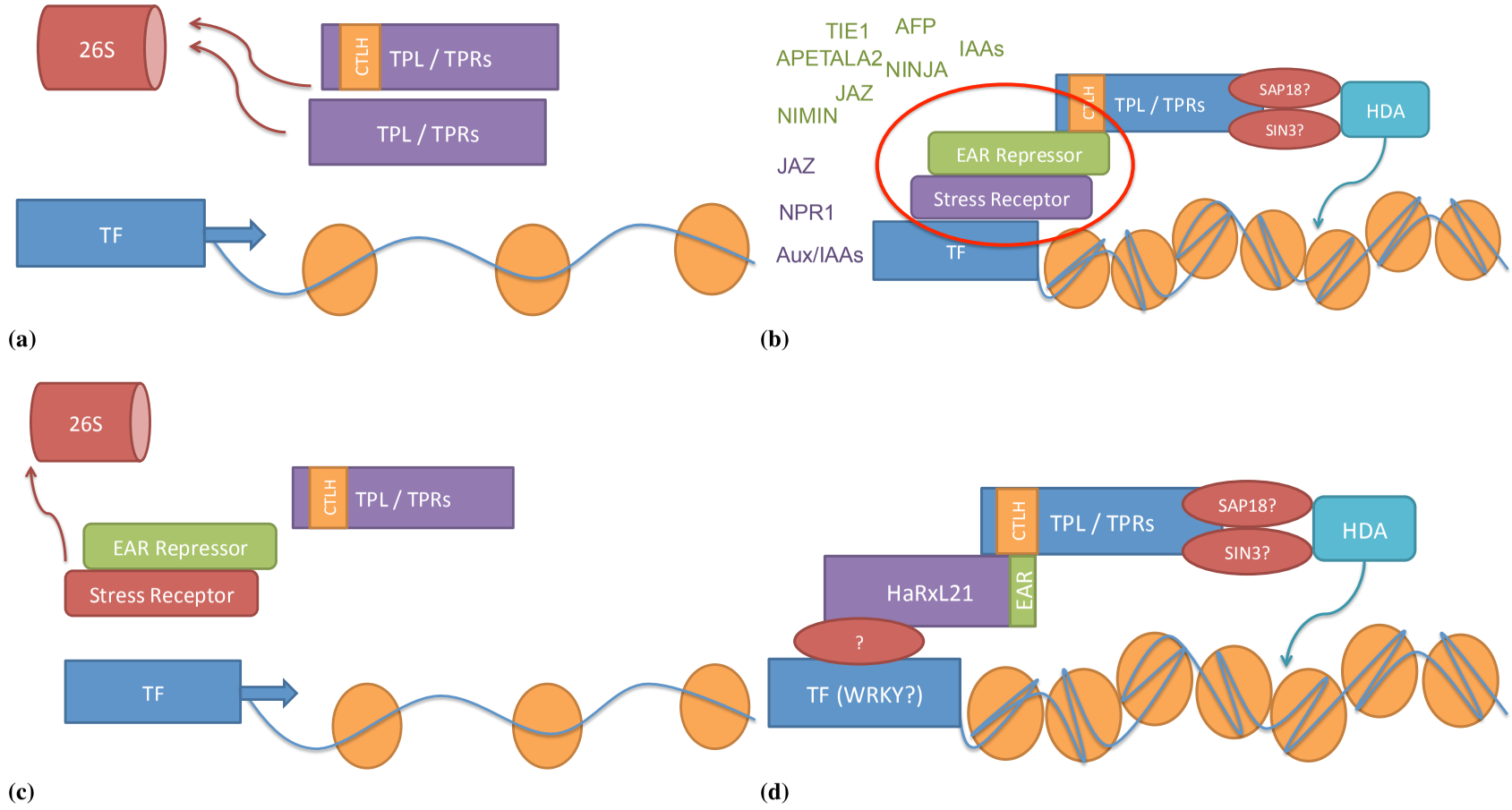


Figure 6.3: Model for the action of TPL and utilisation by HaRxL21.

A potential model for the action of TPL and HaRxL21. (a) 'Inactive' TPL is turned over by the 26S proteasome, as observed when the CTLH domain is removed. (b) TPL is recruited to transcription initiation sites by EAR-motif containing repressor proteins. Examples of EAR repressors and stress receptors are shown. (c) Stress receptors are degraded or removed in the presence of stress (for example due to hormone biosynthesis) therefore releasing TPL. (d) HaRxL21 binds the CTLH domain of TPL via its C-terminal EAR motif, resulting in changes to host transcription. Possible targets include WRKY or ATH transcription factors.

Chapter 7

General Discussion

HaRxL21 expression *in plants* has been found to cause an increase in host susceptibility to the biotophic pathogen *Hpa*, a hemibiotroph (*P. syringae* Fabro et al. (2011); this study) and the necrotroph *B. cinerea*. This is unusual considering how plants employ different defense pathways against these different pathogen strategies (Glazebrook, 2005). The aim of the work here has been to characterise this effector and the mechanism by which it is able to enhance susceptibility to these diverse pathogens.

HaRxL21 was found by Mukhtar et al. (2011) to interact with four proteins; SWAP, TCP14, OBE1 and TOPLESS (TPL). Of these four host targets, interaction with TCP14 was not reproduced here by Y2H, and *in planta* verification of SWAP and OBE1 interactions was questionable; no interaction was detected with SWAP by BIFC and interaction between HaRxL21 and OBE1 was found to occur outside of the nucleus. HaRxL21 has been found here to interact with TPL, an interaction which is mediated by the C-terminal EAR motif of HaRxL21 and the CTLH domain of TPL. This mechanism of interaction utilised by HaRxL21 therefore mimics the mechanism by which TPL and TPL-related proteins (TPRs) interact with plant proteins (Long et al., 2006; Causier et al., 2012). TPL as a host target is unique amongst described effector host targets; despite extensive study of effector protein function and host targets in *P. syringae* there has been no reported interaction with TPL or TPRs (Block et al., 2008; Mukhtar et al., 2011).

None of the other EAR motif-containing *Hpa* effectors tested here showed interaction with TPL, which shows the unique mode of action by HaRxL21. However it is also possible that these were false negatives and that these results also highlight restrictions of using Y2H for detection of protein-protein interactions. For example the interaction between the HaRxL21 Cala2 allele with TPL was not picked up until tested by BIFC and also CO-IP, possibly due to differential protein folding which only occurs in the yeast system.

This result highlights the possibility that interacting targets of other alleles of pathogen effectors may not have been identified by Mukhtar et al. (2011), and further work using *in planta* verification methods such as BIFC or pull downs may reveal additional genuine interactions.

Previous work (Long et al., 2006; Zhu et al., 2010; Pauwels et al., 2010; Causier et al., 2012; Krogan et al., 2012; Tao et al., 2013) has shown that TPL and TPRs are involved in a wide array of processes within *A. thaliana* and that this mechanism shows evolutionary conservation in plants (Causier et al., 2012). In *A. thaliana*, plants which have *TPL* and its closest homologues *TPR1* and *TPR4* knocked out show enhanced susceptibility to *P. syringae* (Zhu et al., 2010) and have been found here to also show enhanced susceptibility to *Hpa* and *B. cinerea*. These data have confirmed the pivotal role of TPL and TPRs at the core of plant immunity and highlighted that they are a key potential target for pathogen effector proteins.

Transcriptional regulation by chromatin remodelling has been implicated in response to multiple stresses; particularly the role of histone deacetylases which deacetylate chromatin and therefore repress gene expression (Wu et al., 2008; Van Den Burg and Takken, 2009; Chen and Wu, 2010; Choi et al., 2012). The mechanism by which TPL brings about transcriptional repression has been linked to chromatin remodelling (Kagale and Rozwadowski, 2011) and in particular, TPL has been shown to interact with HDA19 by BIFC and pull downs (Krogan et al., 2012) although this may be an indirect interaction mediated by other proteins. Transcriptional regulation by TPL has sparked a great deal of interest and targets for TPL binding are being rapidly discovered. Hormone signalling pathways show strikingly similar mechanisms of transcriptional regulation; this mechanism utilises a co-repressor protein which is degraded in the presence of a phytohormone, which in turn interacts with an EAR-motif containing protein that recruits TPL (Weigel et al., 2005; Thines et al., 2007; Wu et al., 2012; Pauwels et al., 2010; Pérez and Goossens, 2013). Although the EAR motif remains a constant feature in all described complexes, sometimes only one protein is required, for example in circadian transcription where TPL has been shown to interact with PSEUDO RESPONSE REGULATOR (PRR) proteins that contain both an EAR motif and a DNA binding domain for directly targeting the promoter region of the target gene (Wang et al., 2013). The manipulation of this mechanism by a pathogen effector highlights the fact that specificity of gene regulation is not determined by TPL and HDAs alone but by the structure of the complex which is recruited either directly to DNA or to a transcription factor. It is possible that there are other proteins yet to be identified which interact with histone deacetylases and control gene expression in this way, also providing potential targets for manipulation by pathogen effectors.

The mechanism by which HaRxL21 alters the host immune system has begun to be unravelled. It is likely that HaRxL21 mimics the action of plant EAR motif-containing repressor proteins and recruits TPL to sites of transcriptional initiation to bring about repression through histone deacetylation. In this work it has been shown that the stability of TPL is increased in the presence of HaRxL21. This mirrors the increase in stability observed when TPL interacts with host-derived EAR motif-containing proteins (shown by comparison with TPLΔCTLH which does not interact with EAR motif-containing proteins). Post translational modification of TPL caused by interaction with EAR motif-containing proteins has not been well characterised, although exploration of potential for post translational modification of TPL has begun (Kagale and Rozwadowski, 2011). The aim of future work arising for the work reported here will be to determine whether there is a difference in post translational modification of TPL and TPLΔCTLH, in addition to determining whether HaRxL21 causes post translational modification of TPL upon interaction.

The mechanism of action employed by HaRxL21 may be similar to that of the EAR motif containing *Xanthomonas campestris* effector XopD (Kim et al., 2008; Canonne et al., 2011) which has been shown to target MYB30 and alter host transcription. XopD effectors have been found in *Xanthomonas*, *Acidovorax* and *Pseudomonas* species (Figure7.1) and a conserved feature across these proteins is the presence of at least one EAR motif. Due to the presence of the EAR motif, it is therefore likely that XopD manipulates host transcription by recruiting TPL to transcription initiation sites, consequently bringing about repression through histone modification. XopD and relatives have currently not been shown to interact with TPL, although this seems a likely hypothesis. These effectors represent a potential for exploring transcriptional reprogramming by effectors from a range of pathogens. It would be interesting to see whether these related effector proteins have the same host targets or whether other aspects of the effector structure allow different transcription factor targets but utilisation of the same mechanism.

Transcriptional effects of HaRxL21 expression *in planta* have given strong indications that plant hormone signalling pathways are disrupted. Microarray data from uninfected tissue prior to *B. cinerea* infection showed an up regulation of genes involved in ABA signalling, concordant with data from microarrays on SA treated tissue which showed an over-representation of transcription factor binding motifs involved in ABA signalling in genes which responded more to SA treatment in the presence of HaRxL21. This effect could be due to either an increase in ABA levels in the tissue of these plants or an increased sensitivity to the hormone. The phytopathogen *P. syringae* perturbs host defences by manipulating the antagonism between ABA and SA signalling (de Torres-Zabala et al., 2007, 2009). It is possible that this strategy is also employed by *Hpa* and that HaRxL21

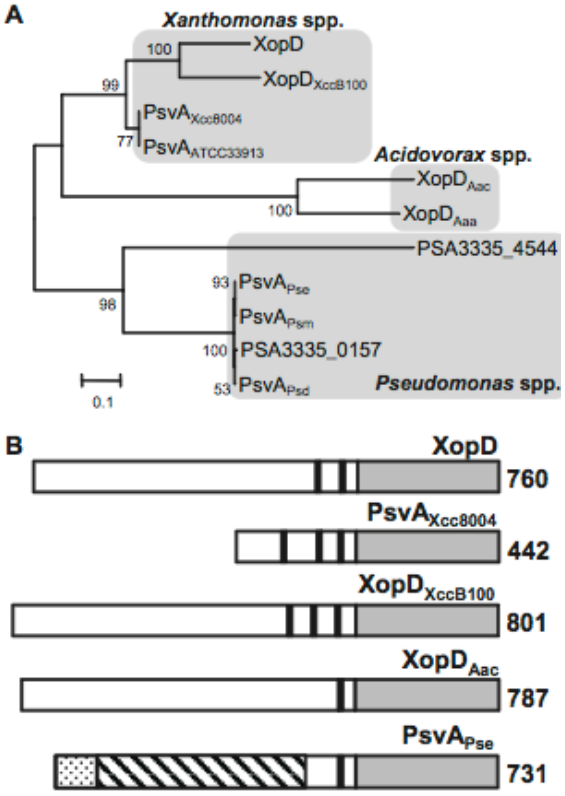


Figure 7.1: XopD Effectors

(A) Phylogenetic tree and (B) Domain structure of XopD and XopD like proteins from *Xanthomonas campestris* pv. *campestris* (Xcc), *P. syringae* pv. *eriobotryae* (Pse) and *Acidovorax avenae* ssp. *citrulli* (Aac). Black bars represent putative EAR motifs. From (Kim et al., 2011).

contributes to this effect.

Genes which responded less in response to SA treatment in *A. thaliana* plants expressing 35S::HaRxL21 showed an over-representation of the transcription factor binding motif 'W Box NPR1' (TTGAC) which occurs in the promoter region of *NPR1* and has been linked to binding by WRKY transcription factors (Yu et al., 2001). This suggests some specificity in the gene expression changes altered by HaRxL21. A role for WRKY transcription factors in negatively regulating ABA signalling has been implicated by Xie et al. (2005); some WRKY transcription factors which are induced by ABA then function to repress ABA signalling. It is possible that HaRxL21 is interfering with this feedback loop and causing the enhanced ABA signalling which is observed in plants prior to *B. cinerea* infection. In addition, the involvement of more WRKY transcription factors in regulation of ABA signalling has been found (Antoni et al., 2011; Liu et al., 2012) and it can be postulated that repression of one may in turn relieve repression of a downstream response.

It is also possible however, that HaRxL21 has multiple transcription factor targets. It may be the case that suppression of SA signalling is mediated through a different target

to that which enhances ABA signalling, rather than a direct consequence of this effector manipulating the antagonism between these pathways.

Future work for this project centres around the characterisation of *A. thaliana* HA::HaRxL21 and HA::HaRxL21ΔEAR plants. Plants expressing HA::HaRxL21 have so far been found not to show the strongly enhanced susceptibility phenotype to *Hpa* which is observed in the 35S::HaRxL21 plants previously used. The possible implications of this centre around the fact that these plants are expressing a tagged protein, unlike HaRxL21a-c. Research here has been focused on the EAR motif at the C-terminus of the protein and therefore screens such as BIFC and Y2H have been performed using N-terminal tags. It is possible that additional interactions which are important for the pathogenicity phenotype have therefore been missed. Stellberger et al. (2010) performed a study of the Varicella Zoster Virus interactome, in which they used Y2H vectors that allowed for C-terminal fusions of DNA binding and activation domains in addition to the usual N-terminal fusions. Using all of the four resulting vector combinations (NN, CC, CN, NC) yielded twice as many interactions compared to NN alone, therefore their screen had fewer false negatives. This system may allow screening for interactions that occur near the N-terminus of HaRxL21, potentially interactions which target the TPL repressor complex to a region of transcriptional activation.

Considering the effector roadmap proposed by Alfano (2009), the study of HaRxL21 and its host targets has given insights into the importance of TPL and TPRs in defense against a diverse range of pathogens. In addition, starting to decipher the mechanism by which HaRxL21 manipulates its host target has given clues as to the biochemical mechanism by which TPL is involved in transcriptional regulation in the host.

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